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STUDIES ON A CELL-DIVISION-CYCLE MUTATOR MUTANT OF *SACCHAROMYCES CEREVISIAE*

by

© Susan Pamela STEWART

A THESIS

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ABSTRACT

The phenotype of a temperature-sensitive cell-division-cycle mutant of *Saccharomyces cerevisiae*, *mut7*, which exhibits high spontaneous mutability at the permissive temperature, was studied in order to elucidate the cause of mutability in this mutant. At the restrictive temperature, 36°C, cultures of *mut7* strains appear morphologically homogeneous, arresting at the "dumbbell" (mother cell with large attached bud) cell-division-cycle terminal phenotype. Because the "dumbbell" terminal phenotype is indicative of a defect in DNA synthesis and nuclear division, *mut7* was of interest in the identification of a potential DNA replication source of spontaneous mutagenesis in *S. cerevisiae*.

DNA specific fluorescent staining revealed the presence of a single, unmigrated nucleus in the mother cell of almost all (83.5%) *mut7* "dumbbell" cells. The time of the required action of the *mut7* gene product was determined by time-lapse photomicroscopy at the restrictive temperature to occur early in the cell cycle, about midway through G1 phase.

Nucleic acid synthesis studies in cultures of *mut7* and wild-type strains at the restrictive temperature indicated that *mut7* is probably defective in the initiation of DNA synthesis at the restrictive temperature. This interpretation is consistent with the estimation of a G1 amount of DNA in each *mut7* "dumbbell" cell. The failure to initiate new rounds of DNA synthesis may be attributed to a

DNA synthesis defect and not to a defect in some other cell cycle event because the high spontaneous mutability of *mut7* strains implies a close association of the *mut7* gene product with DNA replication.

Complementation testing and allelism studies confirm that the *mut7* allele appears to define a previously undescribed cell-division-cycle locus.

Fluctuation data indicated that the spontaneous mutation rate of *mut7* strains was enhanced at the semi-restrictive temperature, 30°C, compared to the permissive temperature, 25°C; this effect was demonstrated at 2 sites, *his1-7* (locus) and *lys1-1* (locus and suppressor). The strongest effect was observed at the *lys1* locus where a 5-fold increase in the spontaneous mutation rate was observed. Under the same conditions, the spontaneous mutation rate of wild-type increased slightly, but this was not significant. Compared to wild-type, *mut7* strains at 30°C had 3-fold to 12-fold higher spontaneous mutation rates, depending on the site tested.

The results are most consistent with a model for *mut7* action where the inhibition of the initiation of DNA synthesis in *mut7* strains indirectly enhances the spontaneous mutation rate.

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INTRODUCTION

Spontaneous mutagenesis in *Saccharomyces cerevisiae* has been studied by the isolation and characterization of two types of mutants; those which are deficient in the repair of radiation induced damage (the *rad* mutants) (reviewed in Lawrence, 1982) and those which have been selected directly for their high spontaneous mutability (the *mut* mutants) (von Borstel *et al.*, 1973; Hastings *et al.*, 1976). Unlike the procaryotes, bacteriophage T4 (reviewed in Drake, 1973) and *Escherichia coli* (reviewed in Cox, 1976), mutants defective in the fidelity of DNA replication have not been identified in *S. cerevisiae*. The studies presented in this thesis describe a potential DNA replication mutant of *S. cerevisiae* which exhibits high spontaneous mutability. This mutant, *mut7*, was originally isolated by von Borstel *et al.* (1973) by selecting directly for mutants exhibiting high spontaneous mutation rates (mutators).

Isolation of Mutator Mutants

Mutants exhibiting high spontaneous mutation rates (mutators) were selected directly by a multi-step enrichment procedure following EMS mutagenesis (von Borstel et al, 1973). Colonies exhibiting an enhanced frequency of white papillations in a red (*ade2-1*) strain background were selected as potential mutators in the first step of the procedure. White papillations could be due to several different mutational events including reversion of the

ade2-1 ochre nonsense mutation, forward mutation at other adenine loci, and forward mutation at ochre nonsense suppressor loci. High frequency papillating strains were subsequently tested for reversion of the *lys1-1* mutation (locus revertants and ochre nonsense suppressor mutants) in limiting lysine medium and for forward mutation to p-fluorophenlyalanine resistance. Strains showing at least a 2-fold increase in spontaneous mutation frequency were retained for further study. Analysis of the mutator mutants of this collection has defined 10 loci, designated *MUT1* through *MUT10* (von Borstel *et al.*, 1973; Hastings *et al.*, 1976; Ord, M.Sc. Thesis, University of Alberta, 1980; S.K. Quah, unpublished data).

The extent of the mutator effect conferred by these alleles varies with the spontaneous mutation test system used. For *mut1* through *MUT6*, *mut9* and *mut10* the strongest mutator effects (up to 25-fold greater than wild-type) are limited to mutation at ochre nonsense suppressor loci (von Borstel *et al.*, 1973; Hastings *et al.*, 1976); while the locus reversion rates vary only up to 5-fold greater than wild-type, depending on the test system used (von Borstel *et al.*, 1973; Hastings *et al.*, 1976). The mutator mutant, *mut7*, was isolated as a double mutant combination with another mutator allele, *mut8*, in a single strain. The double mutant combination, *mut7mut8*, was of particular interest since it exhibited the strongest mutator effect of the entire collection (50-fold locus reversion of *his1-7*) and unlike

the other mutators isolated, which tended to have the strongest effects upon suppressor reversion of *lys1-1*, *mut7mut8* conferred a large enhancement upon reversion of the *lys1-1* locus (40-fold; Table 1, S.K. Quah, unpublished data). The strong mutator effect of the *mut7mut8* double mutant combination was later found to be the result of a synergistic interaction between the single mutator mutations. Separation of the mutator mutations *mut7* and *mut8* during genetic crossing to wild-type strains revealed that each single mutator mutant exhibited only weak mutator activity (5-fold for *mut7* and *mut8* at the *lys1-1* locus; 3-fold for *mut7* and 10-fold for *mut8* at the *his1-7* locus; Table 1, S.K. Quah, unpublished data).

Spontaneous Mutagenesis in *S. cerevisiae*

The correlation between spontaneous mutability and radiation sensitivity is a striking feature of an analysis of spontaneous mutagenesis in *S. cerevisiae*. The radiation sensitive alleles, *rad3*, *rad6*, and *rad51*, which define the putative first steps of the UV-induced damage repair pathways (Cox and Game, 1974), all have enhanced spontaneous mutation rates. Furthermore, one of the alleles selected directly for high spontaneous mutability, *mut5*, is an allele of *rad51* (Morrison and Hastings, 1979). The strength of the mutator effect of these radiation sensitive loci is weak, being about 3- to 4-fold greater than wild-type for reversion of *lys1-1* (combined suppressor and locus

TABLE 1. Spontaneous reversion rates to histidine and lysine independence at 25°C in haploid strains bearing combinations of *mut7*, *mut8* and their respective wild-type alelles (S.-K. Quah, unpublished data).

Genotype	Strain	Reversion Rate (x 10^{-8})		
		Lysine		Histidine
		Suppressor	Locus	Locus
mut7	XV732-2B	2.4	2.5	18.9
mut8	XV731-3D	2.1	2.9	77.5
mut7mut8	XV379-28D	5.6	19.9	300.1
MUT$^+$	XV731-14A	1.3	0.4	6.1

reversion) (Brychcy and von Borstel, 1977; Hastings *et al.*, 1976). Of the radiation sensitive alleles which define subsequent steps of the repair pathways, some are mutators while others are not (Brychcy and von Borstel, 1977; von Borstel *et al.*, 1971).

On the other hand, most (seven out of ten) of the mutator alleles, *mut1* through *mut10*, are sensitive to one or more of the DNA damaging agents tested so far. These agents include UV and gamma-radiation, EMS and MMS (Hastings *et al.*, 1976; Nasim and Brychcy, 1979). For example, *mut7* is only moderately sensitive to MMS and *mut8* is not sensitive to any of the DNA damaging agents above (Nasim and Brychcy, 1979).

The correlation of radiation sensitivity with mutator phenotype, combined with the finding that yeast mutators are weak and numerous in comparison to procaryotic mutators (Drake, 1973; Cox, 1976), has led to the proposal that much of spontaneous mutation arises by the mutagenic repair of spontaneous lesions (Hastings *et al.*, 1976). Thus, the mutator phenotype of the radiation sensitive alleles may be explained as due to blocking the error-free repair pathways and thus forcing repair to occur through mutagenic repair pathways.

The isolation of antimutator strains was hypothesized to determine whether or not any part of spontaneous mutation is a result of DNA replication errors (Hastings *et al.*, 1976). Because these strains are hypothesized to have lost

mutagenic repair capacities, the extent of their antimutagenic effect indicates the proportion of spontaneous mutations which result from the mutagenic repair of spontaneous lesions. Furthermore, antimutator strains are predicted by this hypothesis of mutagenic repair pathways to be repair deficient. Two antimutator mutants, *ant1* and *ant2*, were isolated and found to be UV-radiation sensitive as well as affecting the rate of spontaneous mutation (Quah *et al.*, 1980). UV survival curves for various antimutator and repair deficient mutant combinations demonstrated that *ant2* is an allele of *rev3* and that *ant1* is epistatic to *rev3* (Quah *et al.*, 1980). *Rev3* mutants are deficient in UV-induced mutagenesis and are moderately UV sensitive (Lemontt, 1971), falling into the *RAD6* epistasis group (Lawrence and Christensen, 1976). All induced-mutagenesis deficient mutants, which have been studied to date, belong to the *RAD6* epistasis group (reviewed in Lawrence, 1982). *Rad6* mutants have two major phenotypic effects: they are deficient in all types of induced-mutagenesis (UV: Lawrence and Christensen, 1976; chemical: Prakash, 1976), and they are extremely sensitive to DNA damaging agents (reviewed in Lawrence, 1982). Other mutants in the *rad6* epistasis group do not exhibit all aspects of the *rad6* pleiotropic phenotype and therefore, this suggests that *rad6* plays a central role in the function of this repair group (Lawrence, 1982). Other mutants in the *rad6* epistasis group may be generally classified according to their function into two major *RAD6*

dependent processes (Lawrence, 1982). The first group is defective in error-free processes which are required for resistance to DNA damaging agents (for example, *rad18*) and the second group is defective in mutagenic processes which do not contribute substantially to resistance (i.e. *rev3*). Mutants of the first group are phenotypically mutators (von Borstel *et al.*, 1971) presumably because by blocking an error-free repair process, the probability of mutagenic repair is enhanced. Quah *et al.* (1980) have shown that the two antimutator alleles, *ant1* and *ant2*, belong to the second group of mutants which are involved in the mutagenic repair process known to be required for induced mutation.

The antimutator action of *ant2* (allele of *rev3*) is epistatic to the mutator effect of *rad3*, *rad18*, and *rad51*, suggesting that the enhanced mutability of the *rad* mutants is due to a *REV3* dependent process (Quah *et al.*, 1980). However, the enhanced mutability of *mut1*, is dependent on an *ANT1* dependent process since the antimutator action of *ant1* and not *ant2*, is epistatic to the mutator effect of *mut1*. The *ant1ant2* double mutant produces an additive effect on reduction of the spontaneous mutation rate. These data have led to the conclusion that a least 90% of spontaneous mutations are caused by the mutagenic repair of spontaneous lesions by *ANT1* and *REV3* dependent processes (Quah *et al.*, 1980). Thus, the mutagenic repair process, known to be required for induced mutagenesis, is now known to contribute highly to the production of spontaneous mutations.

The dependence of spontaneous mutagenesis on the function of a mutagenic repair process does not necessarily exclude the influence of alterations in replication fidelity on the spontaneous mutation rate. Endogenously produced spontaneous lesions, resulting from defective DNA replication could, theoretically, provide a substrate for mutagenic repair (Hastings *et al.*, 1976). In addition, mitotic pedigree analysis of UV-induced mutagenesis suggests that mutagenic repair may occur by a mechanism involving reduced DNA replication fidelity enabling gap filling to occur opposite template lesions (James and Kilbey, 1977; Kilbey *et al.*, 2978; James *et al.*, 1978). This mechanism is similar to that which was proposed by Witkin (1967) to explain UV-mutagenesis during SOS repair in *E. coli*. An altered form of DNA polymerase I with reduced replication fidelity has been isolated from *E. coli* cells which have been activated for the expression of DNA damage-inducible (SOS) genes (Lackey *et al.*, 1982). This novel form of DNA polymerase I has been implicated in the proposed mechanism of UV-induced mutagenesis during SOS repair because its reduced replication fidelity would enable gap filling to occur opposite template lesions. Because spontaneous mutagenesis and UV-induced mutagenesis in *S. cerevisiae* both require the *REV3* mutagenic repair process, this suggests that studies on the mechanism of UV-induced mutagenesis may provide a useful working model for deducing the mechanisms of spontaneous mutagenesis.

Although alterations in replication fidelity may influence the spontaneous mutation rate through the function of a mutagenic repair pathway, a DNA replication defective mutator mutant such as has been described in the procaryotes has not been observed in *S. cerevisiae*. DNA polymerase mutants of bacteriophage T4 which have mutator or antimutator phenotypes have been identified (reviewed in Drake, 1973; Kornberg, 1980). Their altered spontaneous mutation rates are interpreted to reflect alterations in the ratio of polymerase activity to 3' → 5' exonuclease activity (reviewed in Kornberg, 1980). DNA polymerase I and DNA polymerase III mutator mutants of *E. coli* have been identified but their mutator effects appear to be restricted to frameshift mutagenesis and are weak in comparison to other *E. coli* mutators (reviewed in Cox, 1976; Siegel and Vaccaro, 1978; Savic and Romac, 1982). On the other hand, mutations at the dnaQ locus, whose gene product is implicated as a component of the replication complex (Maki *et al.*, 1983; Horiuchi *et al.*, 1981), confer strong general mutator effects (100- to 2000-fold increase over wild-type: Horiuchi *et al.*, 1978). The 3' → 5' exonuclease activity of DNA polymerase III holoenzyme which is purified from *dnaQ* and *mutD* (probably an allele of *dnaQ*: Cox and Horner, 1983) strains is found to be defective compared to wild-type (Echols *et al.*, 1983). Thus, the importance of the 3' → 5' exonuclease activity, which is associated with DNA polymerase III, for DNA replication fidelity in *E. coli* is

established.

Although a DNA replication defective mutator mutant has not been observed in *S. cerevisiae*, the *mut7* allele may be an example of this type of mutator mutation. Ord (1980) observed the cosegregation of mutator activity and temperature sensitivity (at 36°C) in crosses segregating the *mut7* allele. Coreversion of the mutator phenotype and temperature sensitivity showed that both phenotypes result from the *mut7* mutation. Microscopic examination of a *mut7* culture arrested at the restrictive temperature revealed a homogenous "dumbbell" cellular morphology. Thus, *mut7* was shown to be a cell-division-cycle conditional-lethal mutation. Since the "dumbbell" cellular morphology is indicative of a defect in DNA synthesis or nuclear division (Culotti and Hartwell, 1971), the phenotype of *mut7* suggested that its mutator effect may be associated with defective DNA replication rather than mutagenic repair of spontaneous lesions.

Genetic Analysis of the Cell Division Cycle

The cell-division cycle of *S. cerevisiae* has been analyzed genetically by the isolation and characterization of conditional-lethal temperature-sensitive mutants blocked at specific stages of the cell-division cycle (Hartwell, 1971a; 1971b; 1973; 1976; Hartwell *et al.*, 1970; 1973; Culotti and Hartwell, 1971; Hereford and Hartwell, 1974; Reed, 1980). The analysis of these cell-division-cycle (*cdc*)

mutants has led to the concept of the cell cycle as a sequence of events organized in a dependent temporal sequence, each requiring the expression of specific genes (Hartwell *et al*, 1974).

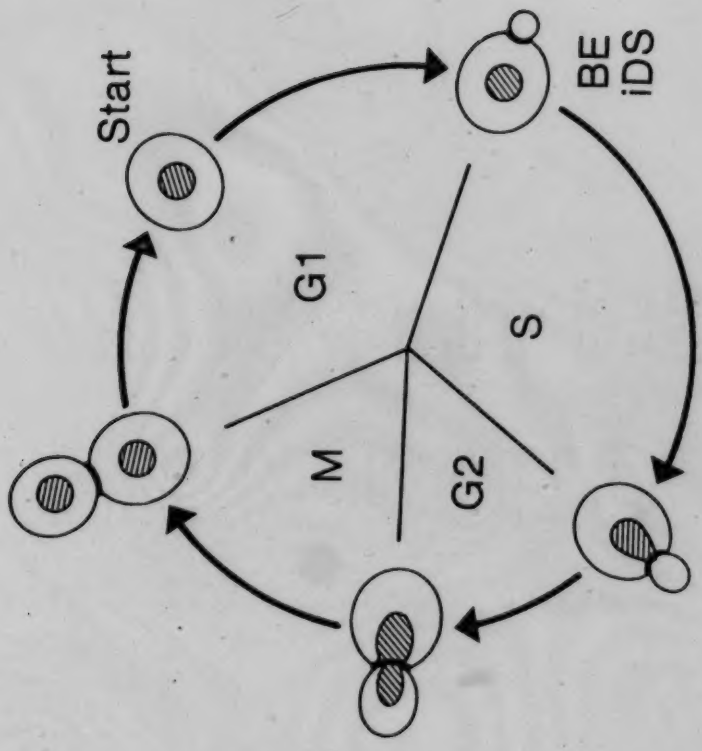
When *cdc* mutants are held at the restrictive temperature, 36°C, each cell ceases normal development at a particular stage, resulting in a morphologically homogeneous population of cells exhibiting a characteristic terminal phenotype for each particular *cdc* mutant. In general, this parameter is locus specific meaning that all the alleles at a single *cdc* locus exhibit the same cellular and nuclear morphology when arrested at the terminal phenotype (Hartwell *et al*, 1973). A second parameter, the execution point, is defined as the last moment in the cell cycle at which the defective *cdc* gene product must act in order to enable the cell to procede into the next cell cycle when being held at the restrictive temperature. Because the execution point is sensitive to the leakiness of a *cdc* mutation, it has been found to be an allele-specific parameter (Hartwell *et al*, 1973). Although the execution points for alleles of the same *cdc* locus are similar, the amount of useful information they provide is questionable, due to the leakiness of these *cdc* mutants. However, they can be used to provide a rough estimate of the time of action of the defective gene product's function in the cell cycle.

The number of *cdc* mutants is increasing continually; however, at the present time there are about 50 defined

loci. The primary defects of these mutants cover all the major landmark events in the cell cycle including "start", bud emergence, initiation of DNA synthesis, elongation of DNA synthesis, nuclear migration, nuclear division, cytokinesis, and cell separation (Figure 1). The first and controlling step in the cell cycle, which is known as "start", determines whether or not a cell will procede into the next cell-division cycle. Absence of an environmental restraint, such as nutrient limitation and mating pheromone, enables the "start" event to be completed, and at this time, the cell is committed to the completion of an entire cell cycle (Hereford and Hartwell, 1974; Hartwell *et al.*, 1974).

Shortly after the "start" event is completed, coincident bud emergence and the initiation of DNA synthesis take place (Hartwell, 1974; Johnston *et al.*, 1980). Once the bud has emerged, it continues to enlarge steadily as the cycle progresses. Thus, the size of the bud provides a means of estimating the position of a particular cell in its division cycle (Hartwell *et al.*, 1970). Observation of the terminal phenotype of double-mutant *cdc* combinations, composed of single *cdc* mutants with distinguishable terminal phenotypes, has allowed the organization of *cdc* gene product functions into dependent pathways containing at least one major branch (reviewed in Hartwell, 1974, 1978). Since the regulation of bud emergence has been shown to occur independently of the initiation and completion of DNA synthesis and nuclear division, the cell cycle has been

Figure 1. The major landmark events of the *Saccharomyces cerevisiae* cell cycle. Abbreviations: BE, bud emergence; iDS, initiation of DNA synthesis.


Start
BE
iDS
G1
S
M
G2

shown to branch into two parallel pathways, the nuclear pathway and the budding pathway (Hartwell, 1971; Hereford and Hartwell, 1974). The sequential function of gene products within the nuclear pathways has been determined in relation to DNA synthesis (Hartwell, 1976); however, the similar terminal phenotype of the *cdc* mutants which are blocked in DNA synthesis and nuclear division has prevented the determination of their temporal sequence by the double-mutant approach.

Dividing the cell cycle into fractions from 0 to 1.0 where 0 is the G1 "start" event, the DNA synthetic (S) phase occupies approximately one-third of the cell cycle from approximately 0.3 to 0.6 (Figure 1) (Lord and Wheals, 1981; Johnston, 1980). *Cdc* mutants blocked in S phase and the subsequent nuclear division event have a characteristic "dumbbell" terminal phenotype which is composed of a mother cell with a large attached bud. "Dumbbell" *cdc* mutants may show a variety of nuclear morphologies ranging from a single nucleus positioned in the mother cell, to a single elongated nucleus positioned at the isthmus between mother cell and bud, to a completely divided nucleus. The relative position of a *cdc* mutant's execution point in the cell cycle is correlated loosely with the nuclear morphology of its terminal phenotype. However, it should be noted that the terminal phenotype may not be correlated directly with a particular stage in the cell cycle since cell cycle events which are not coregulated with the defective event procede

to completion. Thus, bud growth continues in *cdc* mutants which are defective in DNA synthesis and nuclear division yielding the characteristic "dumbbell" morphology.

Many different *cdc* genes are indistinguishable on the basis of terminal phenotype, this being particularly true for the "dumbbell" *cdc* mutants. Moir and Botstein (1982) have determined the order of gene function in the yeast nuclear division pathway using temperature shift experiments involving the temperature-sensitive "dumbbell" *cdc* mutants and a new collection of cold-sensitive "dumbbell" *cdc* mutants. Their results suggest that the nuclear branch of the cell cycle pathway itself contains at least one branch and probably many more. Thus, the nuclear branch of the cell cycle pathway is not simply a dependent series of events but, as expected, involves complex interactions between various *cdc* gene product functions.

Cdc mutants which are known to be defective in DNA synthesis include the initiation mutants *cdc4*, *cdc7*, *cdc2* and possibly *cdc6*, and the elongation mutants *cdc8*, *cdc9*, *cdc21* (Culotti and Hartwell, 1971; Hartwell, 1971a; 1973; 1976; Hereford and Hartwell, 1974), *cdc40* (Kassir and Simchen, 1978), and possibly *cdc16* (Kno *et al.*, 1983). The execution points of the initiation *cdc* mutants range from 0.17 to 0.34, and the elongation *cdc* mutants range from 0.48 to 0.63, expressed as fractions of the cell cycle (Hartwell *et al.*, 1973).

The gene products of only a few of the *cdc* mutants are known. *Cdc8* is defective in single-stranded DNA binding protein (Arendes *et al.*, 1983), *cdc21* is defective in thymidylate synthetase (Game, 1976), and *cdc9* is defective in DNA ligase (Johnston and Nasmyth, 1978).

Cdc4 and *cdc7* have been classified as initiation mutants based on two types of studies. Asynchronous cultures of *cdc4* and *cdc7* undergo a full round of DNA synthesis when shifted to the restrictive temperature, as measured by the incorporation of a radioactive precursor (Hartwell, 1973). Furthermore, synchronous cultures of *cdc4* and *cdc7*, which are shifted to the restrictive temperature after the onset of DNA synthesis, undergo a full round of DNA synthesis (Hartwell, 1973). Examination of the structure of the DNA from cultures of *cdc4* and *cdc7*, which have been arrested at the restrictive temperature, reveals simple linear structures without replication "bubbles" or replication forks (Petes and Newlon, 1974). Thus *cdc4* and *cdc7* appear to be true initiation mutants.

Cdc2 and *cdc6* do not appear to be defective in DNA synthesis based on DNA synthesis kinetics in synchronous and asynchronous cultures (Hartwell, 1973). However, the replication of DNA is dependent upon the prior function of the *cdc2* and *cdc6* gene products. This was determined in reciprocal shift experiments involving the DNA synthesis inhibitor, hydroxyurea (Hartwell, 1976). Conrad and Newlon (1983) examined chromosomal DNA replication in *cdc2* mutants

by incorporation of radioactive precursors in petite derivatives, density transfer experiments and electron microscopy. Their observations suggest that *cdc2* mutants are defective in an aspect of initiation of DNA replication which is common to all chromosomes. At the restrictive temperature, a random fraction (about one-third) of the chromosomes fail to initiate replication but those which do initiate are able to successfully complete replication.

Extrapolation from procaryotic DNA replication studies indicates that there are many replication mutants which have yet to be identified in *S. cerevisiae*. Recent studies involving the screening of *S. cerevisiae* temperature sensitive mutant collections for mutants which are defective in the incorporation of a radioactive precursor into DNA, both *in vivo* (Dumas *et al.*, 1982) and in a permeabilized cell system (Kuo *et al.*, 1983), have identified new conditional-lethal DNA replication mutants. The cell-division-cycle phenotype, if any, of the new mutants isolated by Dumas *et al.* (1982) has not been determined. By screening the temperature-sensitive collection of Hartwell *et al.* (1970), Kuo *et al.* (1983) re-identified *cdc* mutants which are defective in DNA replication and also identified new DNA replication defective mutants which are not *cdc* mutants. In an attempt to identify more DNA replication mutants on the basis of their presumably common "dumbbbell" terminal phenotype, Johnston and Thomas (1982a; 1982b) have isolated the "dumbbell-forming" (*dbf*) mutant series. *Dbf1*

and *dbf2* are defective in the elongation of DNA synthesis and *dbf3* and *dbf4* are defective in the initiation of DNA synthesis. The remaining *dbf* mutants are defective in both RNA and DNA synthesis and may, therefore, be defective in purine or pyrimidine biosynthesis at the restrictive temperature. Following the finding that many of the *dbf* mutants were defective in both RNA and DNA synthesis, Johnston and Thomas (1982a) analyzed the kinetics of DNA synthesis in cultures of the *rna* mutant series. This series was isolated by Hartwell (1967) as temperature-sensitive RNA synthesis mutants. Johnston and Thomas (1982a) found that many *rna* mutants are also defective in DNA synthesis and furthermore that *dbf5* is an allele of *rna3*.

Mutation frequency studies at the permissive temperature indicate that the *cdc7* (Njagi and Kilbey, 1982), *cdc8*, *cdc21* (Newlon *et al.*, 1979), and *dbf1* through *dbf4* (Johnston and Thomas, 1982a) mutants tested, do not differ in mutator activity from wild-type. Because only one allele of each of these *cdc* loci has been tested, it is not possible to conclude that these loci have no effect on the spontaneous mutation rate. Because *cdc21* is defective in thymidylate synthetase, one might expect its mutation rate to be altered as a result of pyrimidine pool imbalance. A high concentration of deoxythymidine monophosphate (dTMP) is mutagenic in *S. cerevisiae*, provided that the cells are permeable to dTMP (Barclay and Little, 1981); however, dTMP starvation has no effect on the mutation rate (Barclay and

Little, 1978). Thus, the absence of any alteration of the mutation rate in a *cdc21* mutant may be a result of the decreased, rather than increased, intracellular concentration of dTMP which would be expected to be found in a mutant lacking thymidylate synthetase. On the other hand, mutation frequency studies indicate that *dbf6*, *dbf5* (*rna3*) and *rna6* are mutator mutants (Johnston and Thomas, 1982a). Because these mutants are defective in both RNA and DNA synthesis, they may be defective in nucleotide synthesis and thus, their mutator phenotype may be the result of nucleotide pool alterations.

Thus, none of the mutants which are specifically inhibited in the initiation or elongation of DNA replication, including *cdc7*, *cdc8*, *cdc21* and *dbf1* through *dbf4* have been found to be mutators in studies to date.

Purpose of Study

The cell-division-cycle phenotype of the *mut7* mutant is unique among the mutator mutants identified in *S. cerevisiae*; none of the *rad* mutants or the other *mut* mutants are also cell-division-cycle mutants. Because the "dumbbell" cellular morphology is indicative of a defect in DNA synthesis or nuclear division, the phenotype of *mut7* suggests that its mutator effect may be associated with defective DNA replication rather than with the mutagenic repair of spontaneous lesions. *Mut7* is of interest, therefore, in the identification of a potential DNA

replication source of spontaneous mutagenesis in *S. cerevisiae*.

In the studies presented here, *mut7* was characterized in the manner of a cell-division-cycle mutant in order to provide information which would be useful in the identification of the mechanism by which *mut7* increases the spontaneous mutation rate. The experimental approach included the determination of the execution point and the nuclear morphology of the terminal phenotype. Because the "dumbbell" terminal phenotype of *mut7* indicated a potential DNA replication defect, the kinetics of nucleic acid synthesis in asynchronous cultures, at both the permissive and restrictive temperatures, were determined by the incorporation of a radioactive precursor into the acid insoluble fraction. Lastly, the mutation rate was measured at both the permissive and semi-restrictive temperatures in order to determine if the mutation rate is enhanced as the *mut7* gene product is partially inactivated.

MATERIALS AND METHODS

Yeast Strains

Tables 2 and 3 list the original haploid strains used in this study, their genotypes and their origin.

Table 4 lists the origin of the diploid strains constructed during the course of this study, and the genotypes of the haploid strains derived from these diploids are listed in Table 5.

Strains grown from spores of the tetrad, SJ701-1, were used for all the cell-division-cycle phenotype studies described here in order to minimize the effects of genetic background differences between strains. The wild-type strains were SJ701-1A and SJ701-1C, and the *mut7* strains were SJ701-1B and SJ701-1D.

Spontaneous mutation studies described here measured the reversion of the alleles *lys1-1* and *his1-7*. The *his1-7* allele is thought to be a missense mutant (Korch and Snow, 1973) and because of its high spontaneous reversion rate and the heterogeneous appearance of the revertant colonies, it is believed to revert through internal missense suppression (von Borstel *et al.*, 1973). The *lys1-1* allele is a suppressible ochre nonsense mutation (Hawthorne, 1969) and can be reverted either by reversion at the *lys1* locus itself or by forward mutation at any of the eight Class I suppressor gene loci (von Borstel *et al.*, 1973). Class I suppressors are tyrosine-inserting, ochre (UAA) nonsense

TABLE 2. Genotype and origin of haploid strains bearing the *cdc* alleles used in this study

Strain	Genotype	Origin
R0428-6C	*α mut7 ade2-1 his1-7 lys1-1 trp5-48 hom3-10*	Robin Ord (1980)
R0428-6D	*a MUT⁺ ade2-1 his1-7 lys1-1 trp5-48 HOM⁺*	Robin Ord (1980)
cdc2	*a cdc2 ade1 ade2 ura1 his7 lys2 tyr1 gal1*	Hartwell et al (1973)
cdc4	*a cdc4 ade1 ade2 ura1 his7 lys2 tyr1 gal1*	Hartwell et al (1973)
cdc7	*a cdc7 ade1 ade2 ura1 his7 lys2 tyr1 gal1*	Hartwell et al (1973)
rna6	*a rna6 ade1 ade2 ura1 his7 lys2 tyr1 gal1*	Hartwell (1967)
dbf5	*α dbf5 ade1 his1 trp2*	Johnston and Thomas (1982)
dbf6 (=rna3)	*α dbf6 ade1 his1 trp2*	Jonhston and Thomas (1982)

Abbreviations: *α* and *a*, complementary mating type alleles; *trp*, *arg*, *his*, *lys*, *ade*, *leu*, *tyr* and *asp*, auxotrophic requirements for tryptophan, arginine, histidine, lysine, adenine, leucine, tyrosine and aspartic acid respectively; *hom*, *ilv* and *aro*, double auxotrophic requirements for methionine and threonine, isoleucine and valine, and phenylalanine and tyrosine respectively; *gal*, inability to ferment galactose; *rna*, inability to synthesize RNA at the restrictive temperature, 36°C; *dbf* represents the formation of the dumbbell cellular morphology at the restrictive temperature, 36°C; and *cdc*, formation of a uniform cell division cycle cellular morphology at the restrictive temperature, 36°C.

TABLE 3. Genotype and origin of haploid strains other than those bearing *cdc*, *rna* and *mut* alleles

Strain	Genotype	Origin
K398-4D	*spoII ura3 ade6 arg6 arg4 aro7 asp5 met14 lys2 pet17 trp1*	Klapholz and Esposito (1982)
416a	*a his4-713 ade2*	Cummins *et al.* (1980)

Abbreviations: see TABLE 2.

TABLE 4. Origin of diploid strains

Diploid	Origin
SJ700	RO428-6C RO428-6D
SJ701	SJ700-1C SJ700-3C
SJ3	416a SJ701-1B
SJ9	SJ3-7A 416a
SJ14	cdc2 SJ9-3B
SJ17	cdc7 SJ9-3B
SJ19	dbf6 SJ9-5B
SJ21	dbf5 SJ9-5B
SJ22	cdc4 SJ9-3B
SJ23	rna6 SJ9-3B
SJ24	K398-4D SJ9-3B
SJ25	SJ24-1A SJ9-3B

TABLE 5. Genotype of haploid strains constructed during the course of this study

Strain	Genotype
SJ700-1C	*a mut7 ade2-1 his1-7 lys1-1 trp5-48 hom3-10*
SJ700-3C	*α MUT⁺ ade2-1 his1-7 lys1-1 trp5-48 hom3-10*
SJ701-1A	*α MUT⁺ ade2-1 his1-7 lys1-1 trp5-48 hom3-10*
SJ701-1B	*α mut7 ade2-1 his1-7 lys1-1 trp5-48 hom3-10*
SJ701-1C	*a MUT⁺ ade2-1 his1-7 lys1-1 trp5-48 hom3-10*
SJ701-1D	*a mut7 ade2-1 his1-7 lys1-1 trp5-48 hom3-10*
SJ3-7A	*α mut7 ade2*
SJ9-2A	*α MUT⁺ ade2*
SJ9-3C	*a MUT⁺ ade2*
SJ9-3B	*α mut7 ade2*
SJ9-5B	*a mut7 ade2*

Abbreviations: see TABLE 2.

codon suppressors which are believed to be genes encoding tyrosine tRNAs (reviewed in Sherman, 1981). Reversion at the *lys1* locus itself results only in lysine independence whereas, a mutation at an ochre nonsense suppressor locus will also result in suppression of another ochre nonsense allele carried by the tester strains in this study, *ade2-1*. Locus revertants can be distinguished from suppressor mutants by the colour of the revertant in medium with a reduced adenine concentration; adjusting the adenine concentration of the medium does not affect the spontaneous mutation rate (Schuller and von Borstel, 1972). Locus revertants will appear red because they remain auxotrophic for adenine and *ade2* mutants accumulate red pigment; suppressor mutants will appear white because they will be prototrophic for adenine.

Media

YEPD is a complex medium for routine growth and viability assays which consists of 1% Bacto-Yeast Extract, 2% Bacto-Peptone, 2% dextrose and 2% Bacto-Agar.

YEPG is a complex medium containing a nonfermentable carbon source (glycerol) that does not support the growth of ρ^- and *pet* mutants. It consists of 1% Bacto-Yeast Extract, 2% Bacto-Peptone, 2% glycerol and 2% Bacto-Agar.

MM is a synthetic minimal medium, which consists of 2% dextrose, 2% Bacto-Agar and 0.67% Bacto-Yeast Nitrogen Base without Amino Acids.

MC (Mortimer Complete) is a synthetic complete medium, which consists of minimal medium supplemented with the following: L-arginine-HCl, L-lysine-HCl, adenine-sulfate, L-methionine, L-tryptophan, uracil and L-histidine-HCl, each at a concentration of 20 mg/liter; L-serine and L-threonine at 375 mg/liter; and L-leucine at 30 mg/liter.

SC is a synthetic complete medium which consists of minimal medium supplemented as for Mortimor Complete medium with the following additional supplements: L-tyrosine and L-isoleucine each at 30 mg/liter; L-phenylalanine at 50 mg/liter; L-glutamic acid and L-aspartic acid at 100 mg/liter; and L-valine at 150 mg/liter. SC was used instead of MC for strains carrying nutritional markers requiring the additional supplements listed above.

Omission media consist of MC or SC lacking one of the supplements. For example, MC-LYS represents MC medium lacking lysine.

FS (Fogel sporulation) medium is used for sporulating diploid strains, and consists of 0.98% potassium acetate, 0.1% dextrose, 0.25% Bacto-Yeast Extract, and 1.5% Bacto-Agar, supplemented as described for MC medium.

Growth limiting liquid media (von Borstel *et al.*, 1971). These media, used for the mutation rate experiments, consist of liquid MC medium with either lysine or histidine (depending on the reversion rate being measured) present in limiting concentration: 1.0 µg/ml for lysine; 0.2 µg/ml for histidine. Limiting lysine medium contains 30% of the usual

amount of adenine (6.6 mg/liter) in order to distinguish locus revertants from suppressor revertants (Schuller and von Borstel, 1974).

Genetic Procedures

Genetic Crosses. Standard procedures for genetic crosses in yeast, including the mating of haploid strains, sporulation of diploid strains and the dissection of asci using a micromanipulator, were used (Sherman *et al.*, 1983).

Replica Plating. Small patches of cells were transferred from a YEPD "master" plate to various types of media by printing with a sterile velvet. This allowed the identification of nutritional genetic markers by printing on omission media, and temperature sensitivity by printing on YEPD and incubating at 37°C.

Complementation Testing. The genotype and mating type of spore clones were determined by complementation. Tester strains and the strains of unknown genotype were streaked across separate YEPD plates, grown overnight and then replica plated crosswise onto YEPD. After incubation overnight, diploids form at the intersections where cells of opposite mating type meet. The genotype of the unknown strains was determined by replica plating the cross-streaked plates on omission media (for auxotrophic makers) or on YEPD followed by incubation at 36°C (for ts markers).

Allelism Testing. To eliminate the possibility of intragenic complementation, diploid strains constructed from

haploid strains of complementing markers were sporulated and about 20 asci were dissected. Independent segregation of the complementing markers indicated their non-allelism.

Mapping Strategy. A strain carrying the unmapped mutation was first crossed to a strain carrying the tightly centromere-linked marker, *met14*. If the unmapped mutation showed centromere linkage, it could then be crossed to a set of centromere tester strains which carried centromere linked markers for all seventeen *S. cerevisiae* chromosomes in order to determine the chromosomal location of the unmapped mutation (Mortimor and Schild, 1981).

Detecting Centromere Linkage by Tetrad Analysis. An exchange between a heterozygous marker and its centromere will result in second division segregation (SDS) for that marker. A gene is considered to be centromere-linked if it shows an SDS frequency significantly less than 0.67. In the case of unordered asci, such as are produced in yeast, a tightly centromere-linked marker such as *met14* may be included in a cross in order to determine the centromere linkage of another marker; the tetratype frequency of a gene against a tightly centromere linked marker then corresponds to the SDS frequency of that gene. The map distance, in CM, of a gene from its centromere can be calculated as approximately half the SDS frequency (Mortimer and Schild, 1981).

Culturing Conditions

The following procedure was used to obtain exponential phase cultures for all experiments. Overnight cultures of the strains to be tested were subcultured 1/250 in 50 ml liquid MC medium and incubated at 26°C in a 250 ml sterile flask, with shaking, for 18 hours. A drop of the overnight culture was spotted onto a YEPD plate and incubated at 37°C overnight in order to determine the temperature-sensitive phenotype. The cells were collected by centrifugation in a clinical centrifuge. The pellet was resuspended in 50 ml liquid medium, the density of this suspension was determined by haemacytometer readings and the suspension was adjusted to the appropriate density by dilution with additional medium, in sterile 50 ml capped Nalgene flasks.

Measurement of Nucleic Acid Synthesis Kinetics

Nucleic acid synthesis was followed by the incorporation of a radioactive precursor, ^{14}C-uracil (New England Nuclear, specific activity 55 mCi/mmol) into the acid insoluble fraction. Exponential phase cultures were prepared as described in MC-URA medium and adjusted to make 25 ml cultures at a density of 2.5 x 10^4 cells/ml. To each culture, 250 µl (25µCi) ^{14}C-uracil was added, and the cultures were swirled and sampled as follows: 100 µl to determine the level or radioactive counts in the culture and 200 µl to determine the level of unincorporated counts which are acid precipitable.

The cultures were incubated at 24°C for 2 hours with shaking in a waterbath to equilibrate the nucleotide pools. At this time, t=0 samples were taken as follows: 800 µl for assay of counts incorporated into DNA, 200 µl for assay of counts incorporated into total nucleic acid and 100 µl for determination of the cell count. Sampling was continued at either 24°C or 36°C with DNA samples taken every 15 minutes, and total nucleic acid samples and cell count samples taken every 30 minutes. At the end of the sampling time, a 100 µl sample was taken to determine that the amount of radioactive label had not become limiting.

For determination of the incorporation of radioactive counts into DNA, 800 µl samples were placed at room temperature in 10 ml capped plastic test tubes containing 800 µl "stop mix" (1M NaOH; 3% Sarkosyl; 50 µg/ml salmon sperm DNA) for approximately 18 hours followed by incubation at 80°C for 15 minutes to hydrolyze the RNA. Samples were cooled for 5 minutes on ice and then precipitated with 4 ml of ice cold 20% TCA. The samples were placed on ice in a coldroom for at least 30 minutes to precipitate the DNA, before collecting on GF/B filters (Whatman).

For determination of the level of incorporation of radioactive counts into both RNA and DNA, 200 µl samples were placed in 10 ml capped plastic test tubes containing 2 ml ice cold 10% TCA and were kept on ice in a coldroom until the end of the sampling period. At this time, the acid precipitated samples were collected on GF/B filters.

Each sample was decanted, under suction, onto a GF/B filter held in a Millipore filtration apparatus. The test tube was rinsed twice with ice cold 5% TCA and the rinses were added to the filter. The filter was removed with Millipore tweezers and pinned to a labelled styrofoam board and dried overnight in a 65°C oven.

For determination of the level of radioactive counts in the culture, 100 µl samples were placed in Eppendorf tubes containing 1 ml ice cold 10% TCA and were left on ice in a coldroom for at least 10 minutes. Following a 5 minute microfugation, 20 µl of the supernatant was sampled directly onto a GF/B filter from a micropipette.

When dry, the filters were placed in scintillation vials with 5 ml toluene fluor (4 l toluene; 0.4 g POPOP; 20 g PPO). Filters were counted in a Beckman scintillation counter and the data were expressed as counts per minute (cpm).

For determination of the cell density of the culture, 100 µl samples were placed in Eppendorf tubes containing 900 µl fixative (0.15 M NaCl, 3.7% formaldehyde) and were refrigerated. The cell density of these samples was determined by counting in a haemacytometer.

Growth and Survival Curve Experiments

Exponential phase cultures were prepared as described and adjusted to make 18 ml cultures at a density of 2.5 x 10^4 cells/ml. The cultures were incubated with shaking at

36°C in a waterbath for 7 hours and samples were removed at regular intervals; every half hour for the first 3 hours, and then every hour for the next 4 hours. One ml samples were placed in 9.0 ml standard lab buffer (1/15 M monobasic potassium phosphate; pH 4.7) The samples were diluted further in standard lab buffer and for each sample, two dilutions were plated on YEPD plates in order to determine the cell viability by colony-forming ability.

Monitoring Cell Number and Cell Morphology

Culturing and sampling conditions were the same as described for the growth and survival curve experiments; the 1.0 ml culture samples were placed in 9.0 ml of fixative (0.15 M NaCl; 3.7% formaldehyde). The fixed samples were observed microscopically for cell number and cell morphology using a haemacytometer. Each plating unit was classified according to the following categories: unbudded cell, cell with small bud (less than 0.3 diameter of mother cell), cell with large bud or "dumbbell" (greater than 0.3 diameter of mother cell) and multi-budded cell. From these data, the cell number, number of plating units and the percentage of cells with large buds ("dumbbells") were determined.

Fluorescent Nuclear Staining and Photomicroscopy

A fluorescent DNA-binding agent, 4',6-diamidino-2-phenylindole (DAPI; Sigma), was used to detect the nuclear morphology of yeast cells according to the staining

procedure of Williamson and Fennel (1975). Exponential phase (15 ml) cultures were prepared as described and adjusted to a density of 1 x 10^7 cells/ml. These cultures were incubated at 36°C for 4 hours with shaking in a waterbath. After incubation, each culture was poured into a large capped test tube with 35 ml of 95% ethanol to give a final ethanol concentration of approximately 70%. The cells were fixed for 30 minutes at room temperature and then collected by centrifugation. The pellet was resuspended in 15 ml sterile distilled H_2O with 600 µl DAPI staining solution (10 µg/ml in distilled H_2O) and incubated overnight at 4°C. One ml aliquots of each culture were microfuged for 5 minutes in Eppendorf tubes, and the pellet was resuspended in 200 µl 40% polyethyleneglycol (PEG) to immobilize the cells during photomicroscopy. Cell clumps were dispersed by sonication (10 sec, 35% power, thin probe).

The stained cells were observed through a Leitz Orthoplan microscope equipped with epi-fluorescence and Ploem optics. Since DAPI requires a 365 nm excitation wavelength, filter type "A", which produces an emission wavelength less than 395 nm, was used. The cells were observed with a 63x oil objective.

For photomicrography, Tri-X (ASA 400) film was exposed for 1.5 minutes and then developed as for ASA 1000 film.

Time Lapse Photomicroscopy

The execution point of *mut7* was determined by the method of Hartwell *et al.* (1973). Cells from an exponential phase culture were spotted onto agar plates prewarmed to 36°C, photographed immediately, and again after 6 hours at 36°C. Approximately 100 cells were observed and scored as to whether they had arrested in the first or second cell cycle. From these data, the execution point was calculated and expressed as a fraction of the cell cycle. The method of computation is as follows (Hartwell *et al.*, 1973):

N = fraction of cells that produce two cells at restrictive temperature

$$Ex = 1 - \log(1+N)/0.301$$

Measurement of Spontaneous Mutation Rates: The 1000-Compartment Fluctuation Test

Measurement of Reversion Frequency. Suspensions of 1 x 10^7 cells/ml were made from single colonies grown for three days on YEPD. From this suspension, 0.5 ml was plated on each of two plates of omission medium (MC-HIS or MC-LYS), to determine the frequency of pre-existing revertants, and 0.24 ml was inoculated into 1200 ml of limiting liquid medium. A 1/10 dilution of the inoculated medium was made and 0.5 ml was plated on each of two YEPD plates to determine the number of viable cells/ml in each flask. The medium was stirred continuously with a sterile magnetic stirrer while 1.0 ml aliquots were collected in ten 100-compartmented culture boxes using a Brewer Automatic Pipetting Machine,

model No. 60453 (Baltimore Biological Laboratory). The calibration of the syringe was checked before and after each experiment by collecting 20 squirts in a 25 ml graduated cylinder. After being filled, each box was sealed with masking tape, placed in Zip-Loc Storage bags in sets of five, and incubated at 25°C (permissive) or 30°C (semi-restrictive for *mut7* strains).

Revertants to lysine (or histidine) independence could continue to grow after the supply of lysine (or histidine) had been depleted and, therefore were capable of forming colonies. After 12 days incubation, the number of revertants in each compartment and the total number of revertant-containing compartments were recorded for each box. The number of cell divisions which occurred before the limiting nutrient was depleted was determined by counting the number of cells/ml in two compartments without revertants in each box according to a prearranged pattern.

Computation of Mutation Rates. The number of revertant colonies in independent compartments was assumed to follow a Poisson distribution. To calculate the reversion rates, the number of compartments without revertant colonies was used. The method of computation was as follows (von Borstel *et al.*, 1971):

> Let N be the number of compartments in an experiment, and N_0 the number of compartments without revertants. From the zeroth term of a Poisson distribution we have
>
> $$e^{-m} = N_0/N$$
>
> where m equals the average number of mutational

events (not mutants) per compartment. Most of these mutational events are due to new mutations arising during the growth of the cells in the limiting medium, but some are due to mutants, present in the inoculum. We correct for this "background" by

$$m_g = m - m_b$$

where m_b is the average number of mutants per compartment in the inoculum (as determined by direct plating), and m_g is the corrected average number, i.e., the mutational events occurring during the growth in the compartments. This can be converted to the mutational events per cell per generation, M, by

$$M = m_g/2C$$

where C is the number of cells per compartment after growth has ceased in the limiting medium. The factor of two in the denominator is necessary because the number of cell generations in the history of a culture is approximately twice the final number of cells. Actually, the proper value for this numerical factor depends upon the point(s) in the cell cycle at which growth is terminated in the limiting medium and also upon the distribution of mutation production over the cell cycle. Since it enters only as a scale factor in all mutation rate calculations, relative mutation rates are unaffected by the value used.

This method for determining mutation rates is due to Luria and Delbruck (1943). The principal advantage of the method is that the results are not affected by many types of selection. Since we only score the presence or absence of a mutational event in a culture, it is clearly irrelevant whether the mutants grow faster or slower than non-mutants.

In those experiments where the mutants are further analyzed into categories the mutation rate may be partitioned by

$$M_i = f_i M$$

where M_i is the mutation rate (per cell per generation) for the ith category and f_i is the fraction of the mutants tested which were found to be in the ith category.

RESULTS

Temperature Sensitive Phenotype of *mut7* Haploid Strains

When exponential phase cultures of wild-type and *mut7* strains were shifted from 25°C to 36°C, the wild-type strains continued to grow at an exponential rate whereas the *mut7* strains ceased growth (Figures 2 and 3). After an initial lag of 1 hour, the wild-type strains, SJ701-1A and SJ701-1C, grew at an exponential rate with a generation time of 1.5 hours. The *mut7* strains, SJ701-1B and SJ701-1D, initially showed cessation of growth for about 2 hours followed by exponential loss of viability, which was measured as colony-forming ability. After 7 hours at the restrictive temperature, 36°C, the *mut7* strains showed about 25% survival. These data established the temperature sensitive phenotype of *mut7* haploid strains and the temperature insensitivity of the genetically related wild-type haploid strains.

Cellular Morphology of *mut7* Haploid Strains at the Restrictive Temperature

When exponential phase cultures of wild-type and *mut7* strains were shifted from 25°C to 36°C, observation of the cellular morphology revealed that the proportion of "dumbbell" cells in the *mut7* culture increased steadily with time of incubation, whereas the proportion of "dumbbell" cells in the wild-type culture did not increase (Figure 4).

Figure 2. Survival of the *mut7* haploid strains, SJ701-1D, after exposure to the restrictive temperature, 36°C.

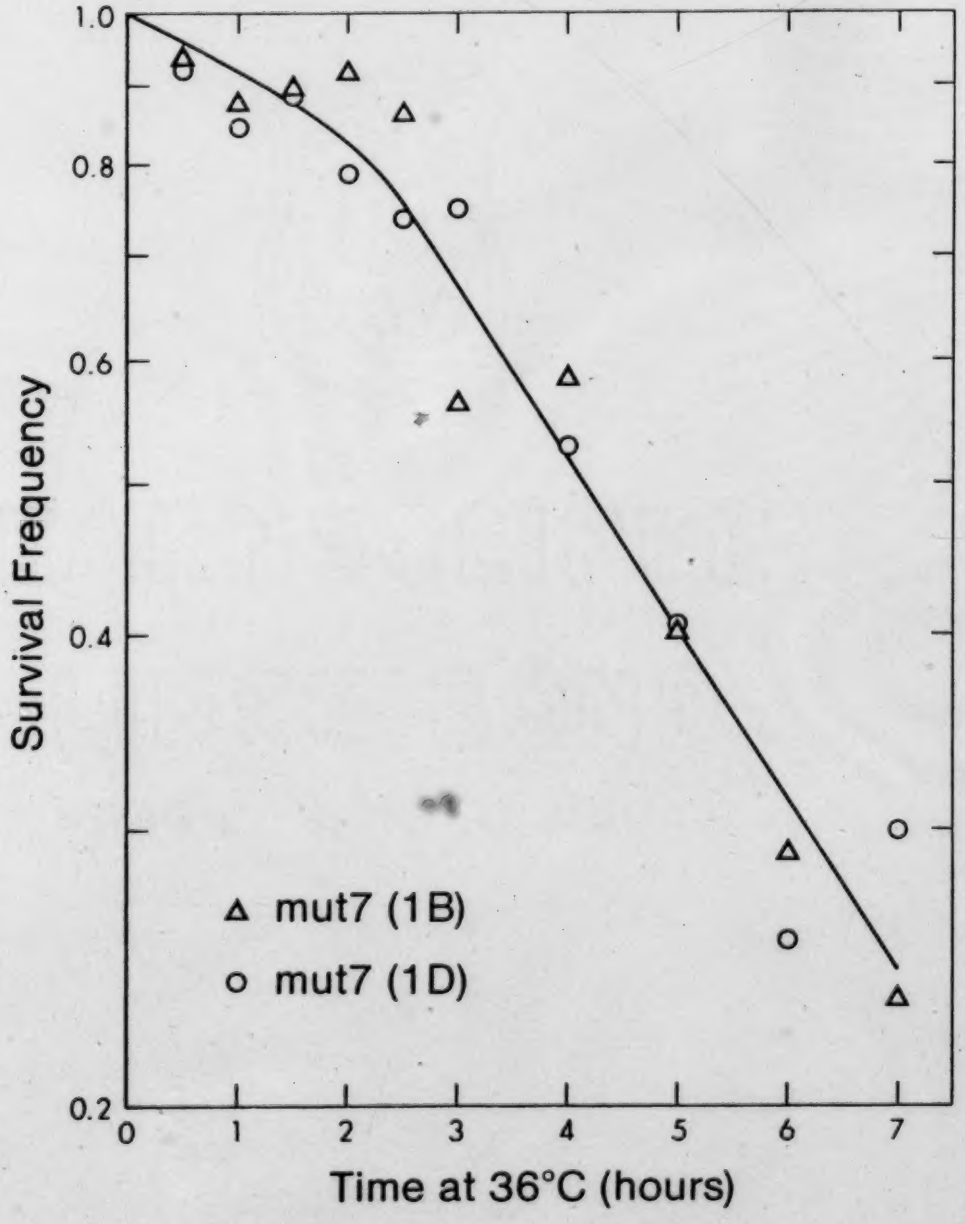

1.0
0.8
0.6
0.4
0.2
Survival Frequency
0
1
2
3
4
5
6
7
Time at 36°C (hours)
mut7 (1B)
mut7 (1D)

Figure 3. Growth of the wild-type haploid strains, SJ01-1A and SJ701-1C, at 36°C.

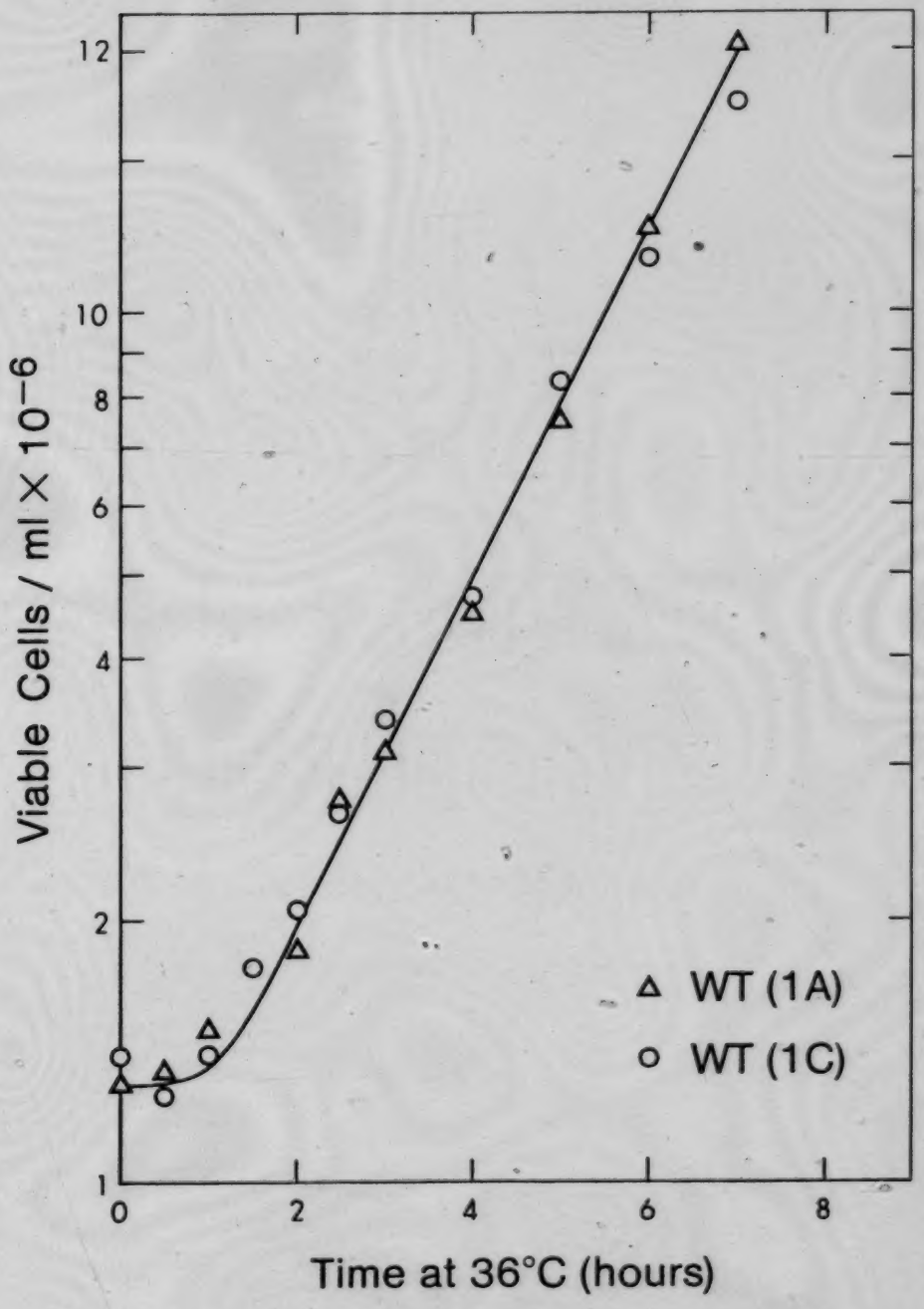
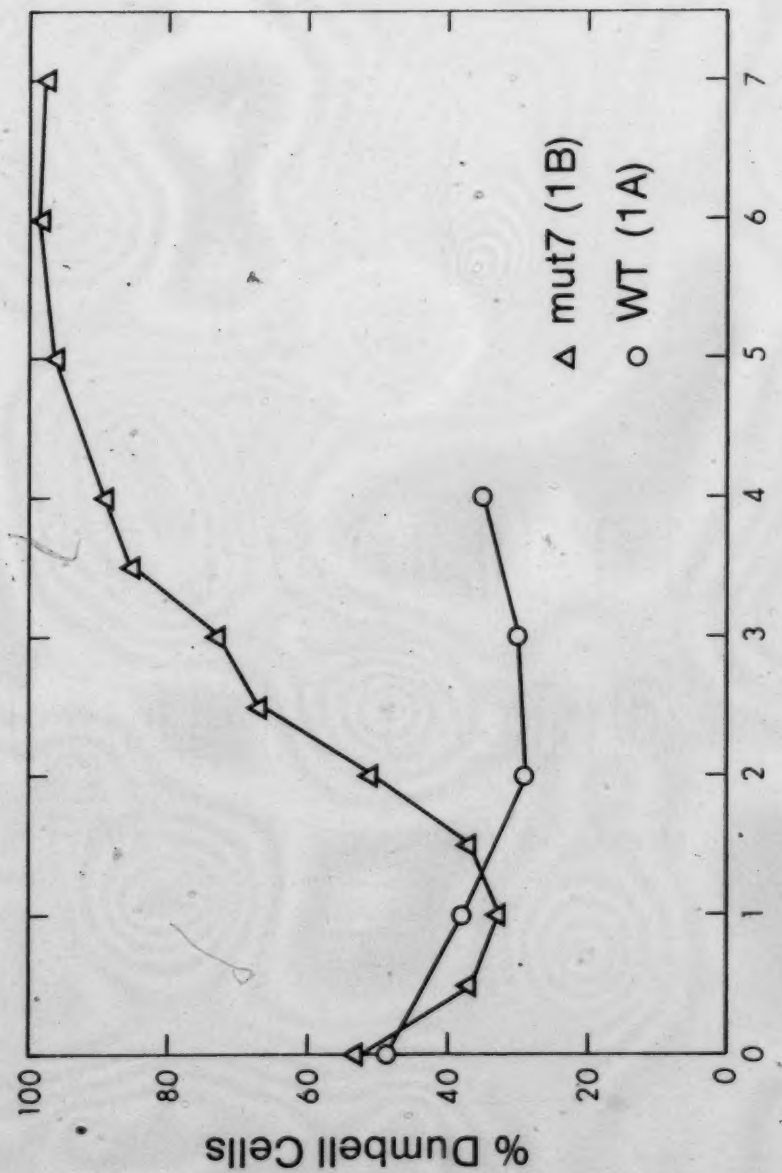

Viable Cells / ml X 10⁻⁶
12
10
8
6
4
2
1
0
2
4
6
8
Time at 36°C (hours)
WT (1A)
WT (1C)

Figure 4. Proportion of "dumbbell" cells in cultures of the wild-type and *mut7* haploid strains, SJ701-1A and SJ701-1B, at 36°C.


% Dumbell Cells
100
80
60
40
20
0
0
1
2
3
4
5
6
7
Time at 36°C (hours)
▲ mut7 (1B)
○ WT (1A)

At the permissive temperature, 25°C, both the wild-type and *mut7* cultures showed about 50% "dumbbell" cells. After a shift to the restrictive temperature, 36°C, the wild-type culture initially showed a steady decline to a stable proportion of 30 to 40% "dumbbell" cells which may reflect the faster growth kinetics at the higher temperature. On the other hand, the proportion of "dumbbell" cells in the *mut7* culture steadily increased until, after 6 hours at the restrictive temperature, greater than 95% of the culture had collected at the "dumbbell" cellular morphology. Thus, *mut7* is a cell division cycle mutant; at the restrictive temperature, *mut7* strains produce morphologically homogeneous cultures exhibiting the "dumbbell" terminal phenotype.

Terminal Phenotype of *mut7* Haploid Strains at the Restrictive Temperature

DNA specific fluorescent straining with DAPI (Williamson and Fennell, 1975) revealed the nuclear morphology of the *mut7* terminal phenotype. A single nucleus, which had not migrated to the isthmus between the mother cell and the bud of each "dumbbell", was present in the mother cell of almost all (83.5%) of the "dumbbell" cells in a culture of the *mut7* strain, SJ701-1B, incubated at the restrictive temperature for 4 hours (Figure 5). Under the same conditions, the wild-type strain, SJ701-1A, showed a variety of cellular and nuclear morphologies as would be

Figure 5. Nuclear and cellular morphology, revealed by DNA specific fluorescent staining, of the wild-type and *mut7* haploid strains, SJ701-1A and SJ701-1B, after 4 hours incubation at 36°C.

WT(1A)

mut7(1B)

expected in an asynchronous, exponential phase culture.

A sample of 124 cells from a *mut7* culture which was arrested at the restrictive temperature, was scored for nuclear and cellular morphology after DAPI staining. A small portion (2.4%) of the sample was arrested at an unbudded cellular morphology whereas, most of the sample (97.6%) had been arrested at a "dumbbell" cellular morphology. Of the "dumbbell" cells, a significant portion (14.0%) showed a nucleus which had migrated to the isthmus and a small portion (2.5%) showed a divided nucleus. However, the majority of "dumbbell" cells (83.5%) showed the presence of a single nucleus which had not migrated to the isthmus. Both the mother cell and the bud of the *mut7* "dumbbell" cell were enlarged in volume in comparison to the wild-type cells under the same conditions (Figure 5). When held for extended periods of the time at the restrictive temperature (8 hours or more), both the mother cell and bud developed an elongated morphology which is similar to that which is observed in wild-type cultures reacting to mating pheromone (data not shown). Thus, although some diversity in the terminal phenotype of *mut7* was found, the majority of cells (82%) in a *mut7* culture, after 4 hours incubation at the restrictive temperature, exhibited the uninucleate, enlarged "dumbbell" morphology.

Execution Point Determination for *mut7* Haploid Strains

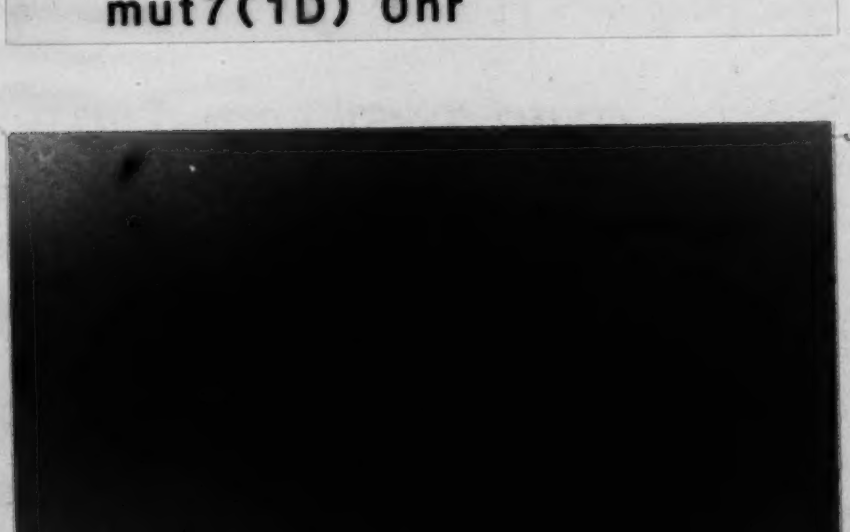
The execution point, which is the time of action for the mutant gene product, was determined for the *mut7* haploid strain, SJ701-1B, by the method and calculations of Hartwell *et al*. (1973). A sample of 105 cells was observed and scored as to whether arrest at the "dumbbell" morphology had occurred in the first or second cell cycle after 6 hours at the restrictive temperature. Most of the culture (82%) were arrested in the second cycle, resulting in the production of two "dumbbell" cells, whereas a smaller portion of the culture (18%) were arrested in the first cycle, resulting in the production of a single "dumbbell" cell. From these data, the execution point of *mut7*, expressed as a fraction of the cell cycle, was calculated to be 0.14. In terms of the landmark events of the cell cycle, this point is midway through the G1 phase. This result was supported by time-lapse photomicroscopy data which revealed that unbudded cells, at the time of the temperature shift, stopped in the first cycle, whereas budded cells (ranging in size from very small to large) stopped in the second cycle after the temperature shift (Figure 6). Thus, the execution point of *mut7* occurred very early in the cell cycle, immediately prior to bud emergence.

Figure 6. Time-lapse photomicroscopy of cells from the *mut7* haploid strains, SJ701-1B and SJ701-1D, at the restrictive temperature, 36°C. Arrows designate cells at the morphological stages which flank the execution point.

mut7(1B) 0hr

mut7(1B) 6hr

mut7(1D) 0hr



mut7(1D) 6hr

Nucleic Acid Synthesis Kinetics in Asynchronous Cultures of *mut7* and wild-type Haploid Strains

The kinetics of RNA and DNA synthesis were determined over several hours at both the permissive and restrictive temperatures in asynchronous cultures of wild-type and *mut7* strains by the method of Johnston and Game (1978).

At the permissive temperature, 25°C, incorporation of radioactive counts into both RNA and DNA increased at an exponential rate in the wild-type and *mut7* strains, SJ701-1A and SJ701-1B (Figures 7 and 8). At the restrictive temperature, 36°C, incorporation of radioactive counts into both RNA and DNA increased at an exponential rate in the wild-type strain, SJ701-1A (Figure 9). However, whereas incorporation of radioactive counts into RNA increased at an exponential rate in the *mut7* strain, SJ701-1B, incorporation into DNA was inhibited relative to the wild-type strain (Figure 10).

The interpretation of the differences in nucleic acid synthesis kinetics between *mut7* and wild-type strains at the restrictive temperature was facilitated by the simultaneous determination, by haemacytometer, of the cell density of these radioactively labelled cultures. The increase in cell density of the exponentially growing *mut7* and wild-type cultures was transiently inhibited (45 to 60 minutes) upon shifting from 25°C to 36°C (Figure 11). Following this period of inhibition the wild-type culture resumed an exponential rate of increase in cell density with a doubling

Figure 7. Nucleic acid synthesis in an asynchronous culture of the wild-type haploid strain, SJ701-1A, at 25°C.

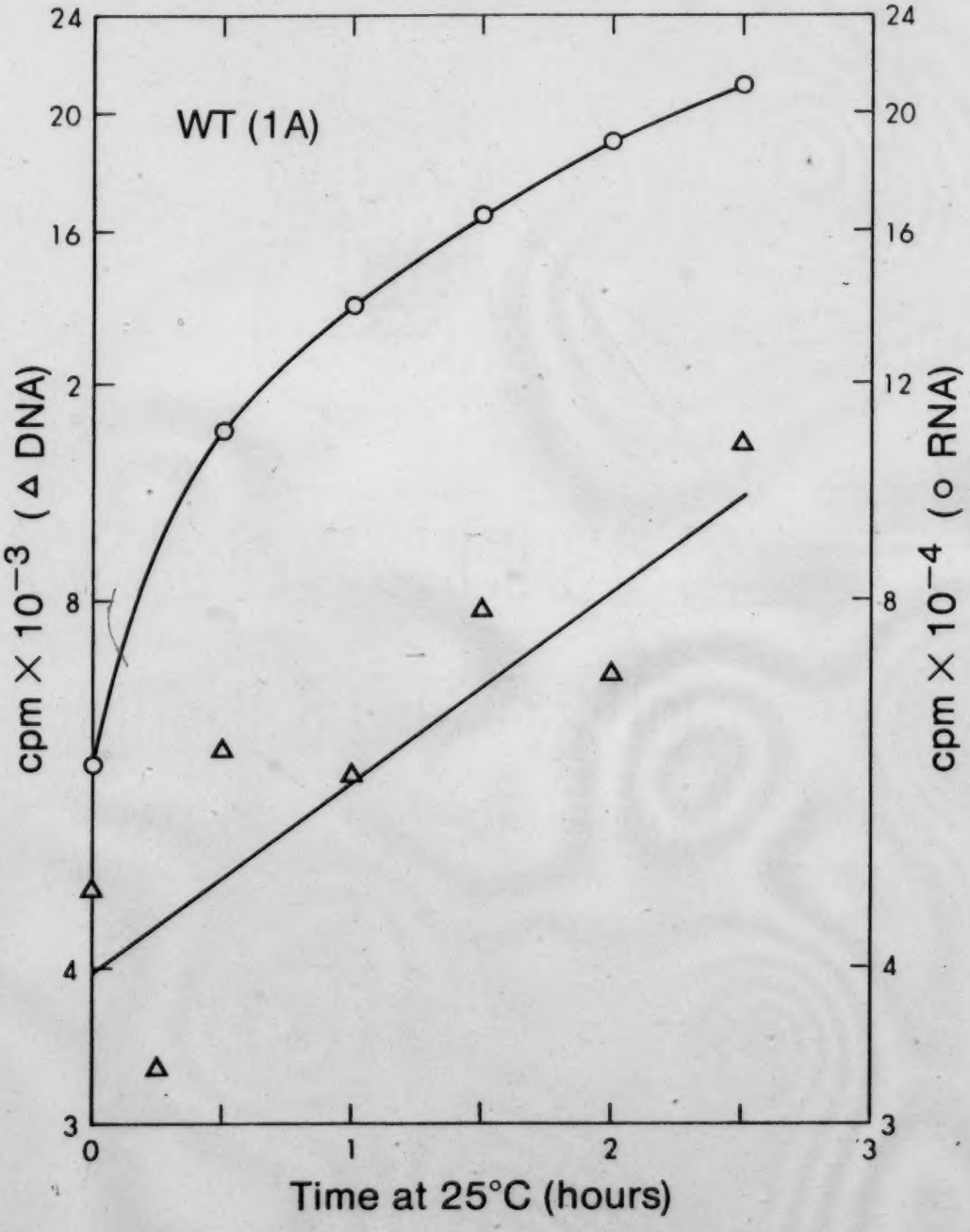
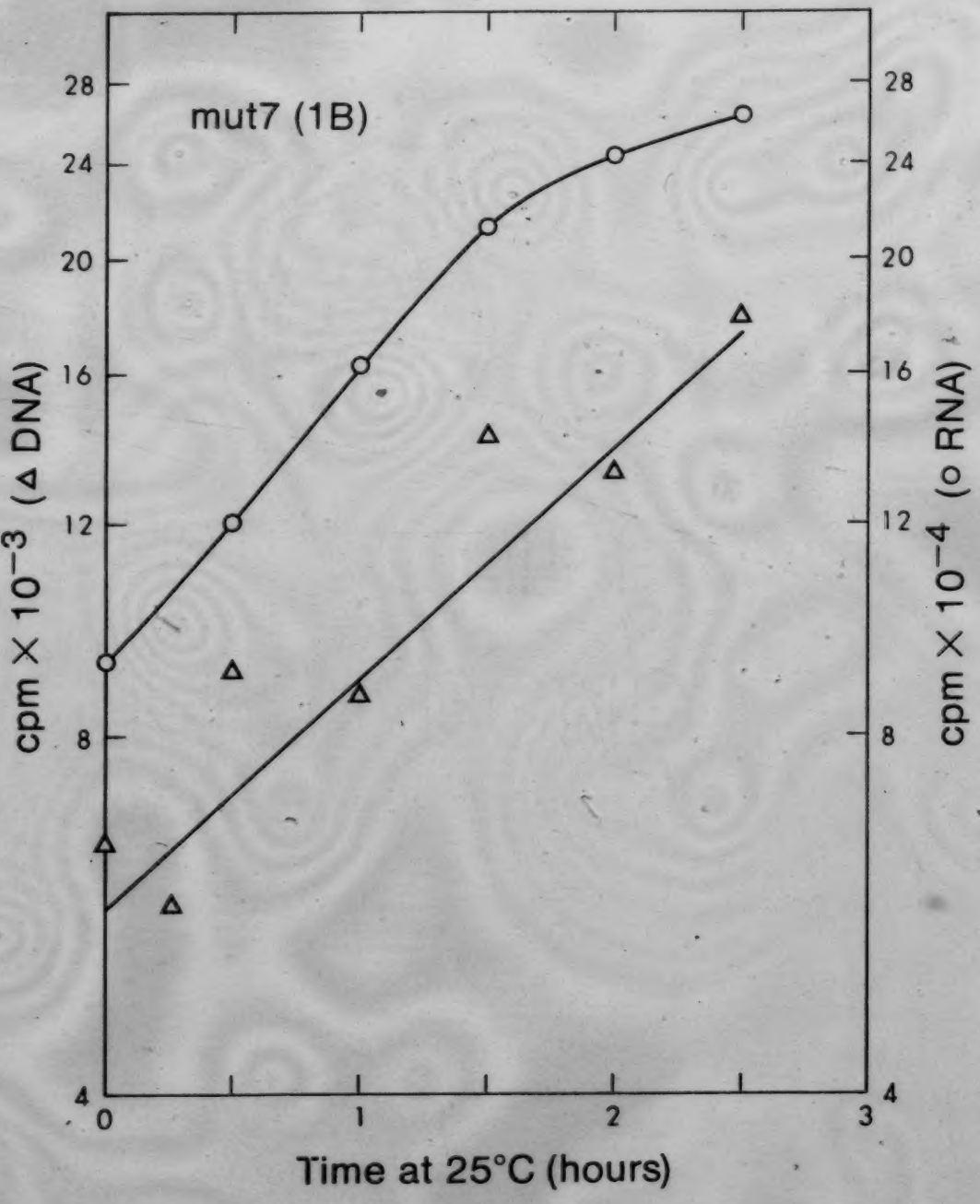

WT (1A)
cpm X 10⁻³ (Δ DNA)
cpm X 10⁻⁴ (o RNA)
Time at 25°C (hours)
24
20
16
2
8
4
3
12
0
1
2
3

Figure 8. Nucleic acid synthesis in an asynchronous culture of the *mut7* haploid strain, SJ701-1B, at the permissive temperature, 25°C.

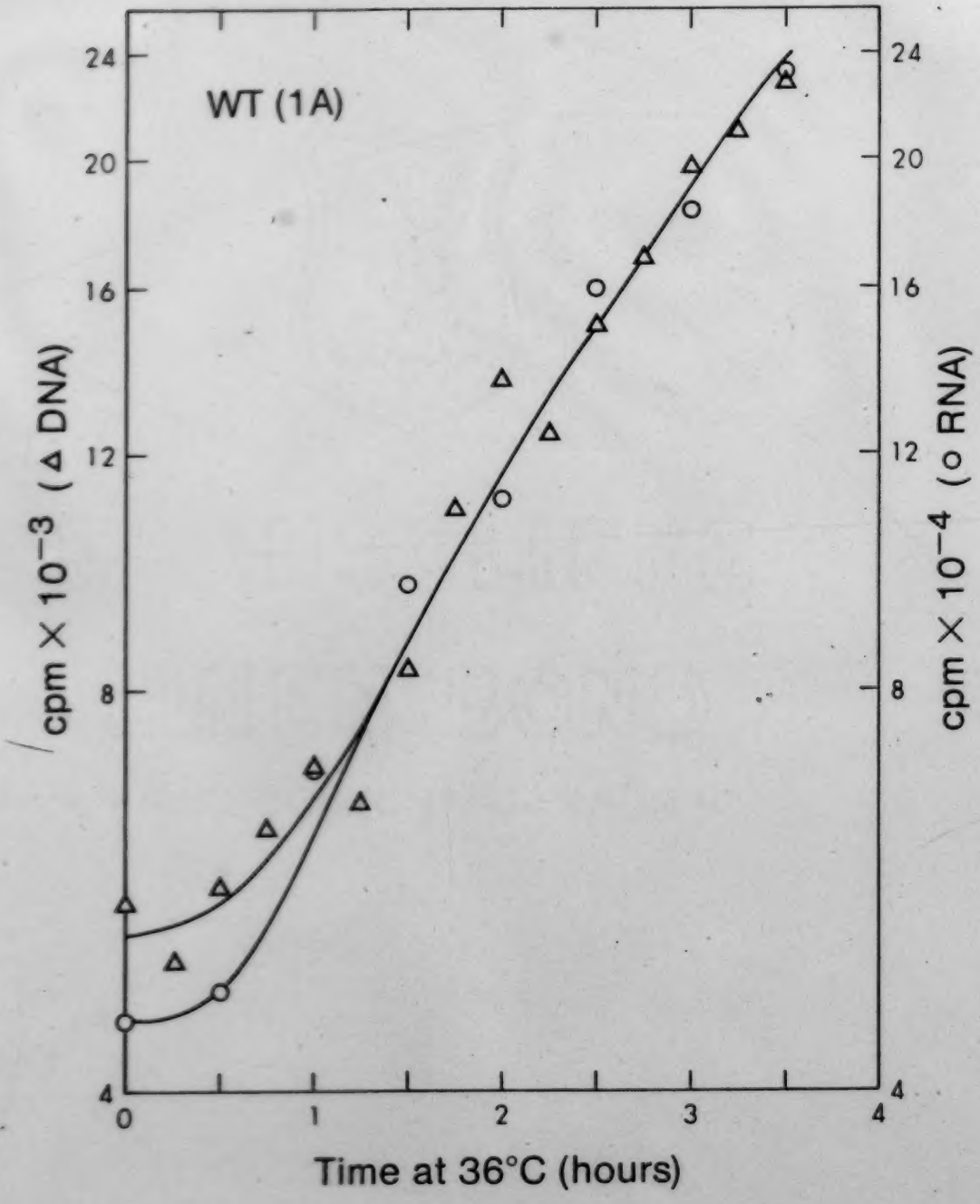

mut7 (1B)
cpm X 10⁻³ (Δ DNA)
cpm X 10⁻⁴ (o RNA)
Time at 25°C (hours)
28
24
20
16
12
8
4
0
1
2
3

time of 1 1/2 hours for the remaining course of the experiment (Figure 11). On the other hand, the *mut7* culture showed an exponential rate of increase in cell density which was approximately half the rate of the increase shown by the wild-type culture under the same conditions (Figure 11). The rate of increase in cell density of the *mut7* culture slowed after about 3 hours at the restrictive temperature by which time the cell density of the *mut7* culture had almost doubled from that at the time of the temperature shift and about 85% of the cells in the culture displayed the "dumbbell" cellular morphology.

The rate of incorporation of radioactive counts into RNA in a *mut7* strain at the restrictive temperature increased exponentially, but at a slower rate than wild-type, throughout the course of the experiment; however, after 2 hours at the restrictive temperature incorporation into RNA in the *mut7* strain slowed slightly relative to the initial rate of incorporation into RNA. The slower rate of RNA synthesis is consistent with the slower rate of increase in cell density also seen in the *mut7* culture compared with the wild-type culture at 36°C (Figure 11). Because RNA synthesis generally continued to increase exponentially while cessation of cell division was occurring, these data indicated than RNA synthesis was uninhibited in a *mut7* strain at the restrictive temperature.

The rate of incorporation of radioactive counts into DNA in the *mut7* strain at the restrictive temperature

Figure 9. Nucleic acid synthesis in an asynchronous culture of the wild-type haploid strain, SJ701-1A, at 36°C.


WT (1A)
cpm X 10⁻³ (Δ DNA)
cpm X 10⁻⁴ (o RNA)
Time at 36°C (hours)
24
20
16
12
8
4
0
1
2
3
4

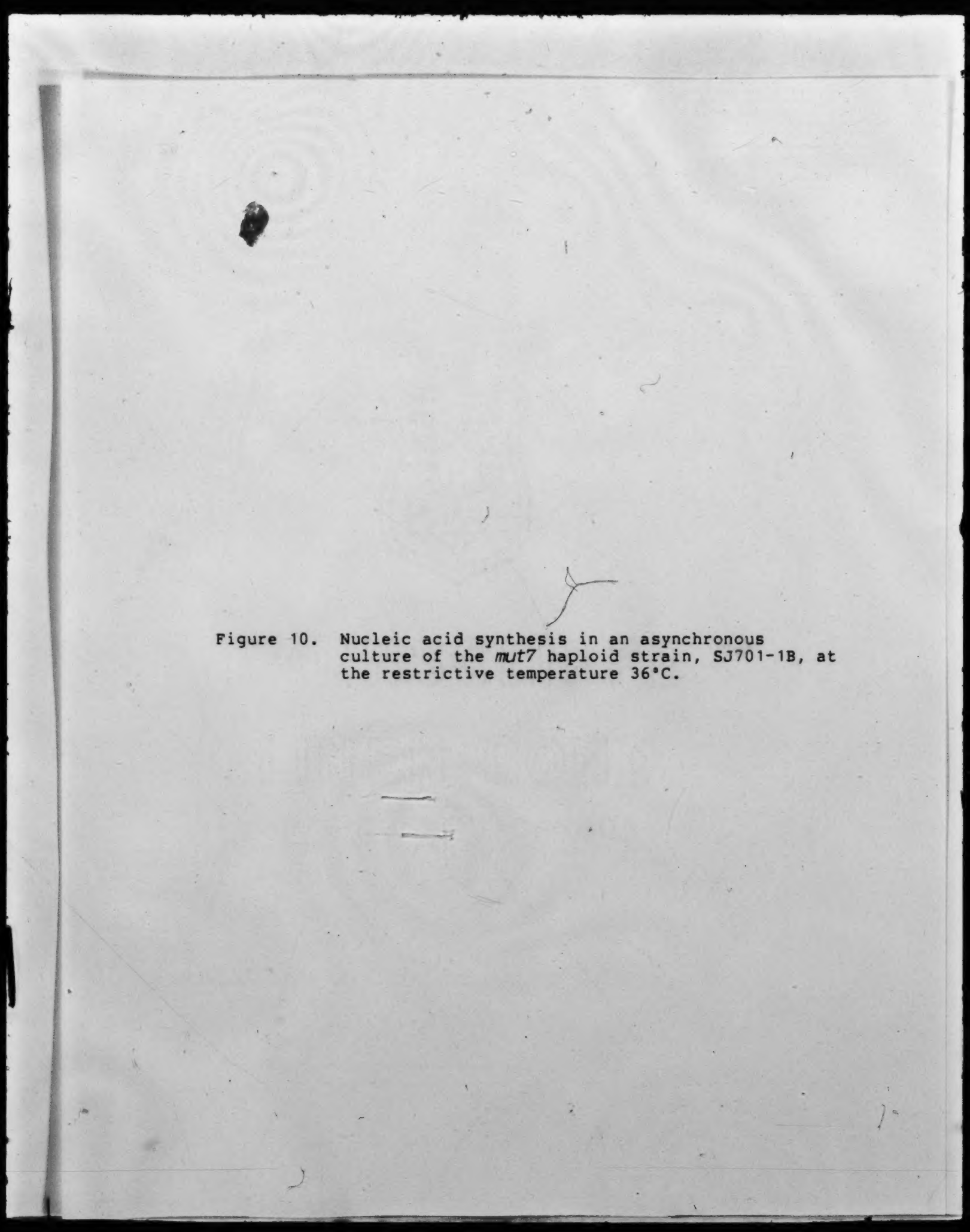
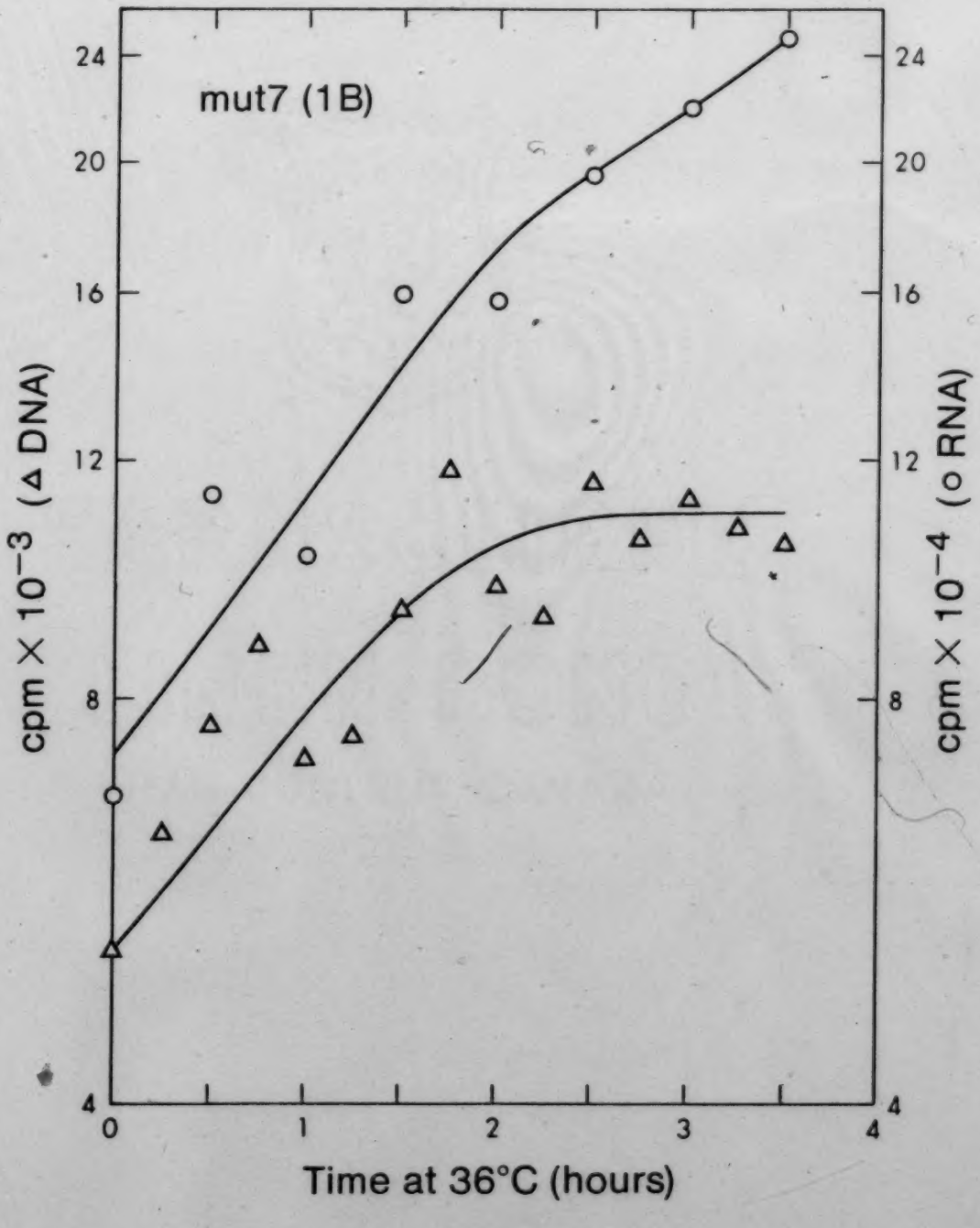


Figure 10. Nucleic acid synthesis in an asynchronous culture of the *mut7* haploid strain, SJ701-1B, at the restrictive temperature 36°C.


mut7 (1B)
cpm X 10⁻³ (Δ DNA)
cpm X 10⁻⁴ (o RNA)
Time at 36°C (hours)
24
20
16
12
8
4
0
1
2
3
4

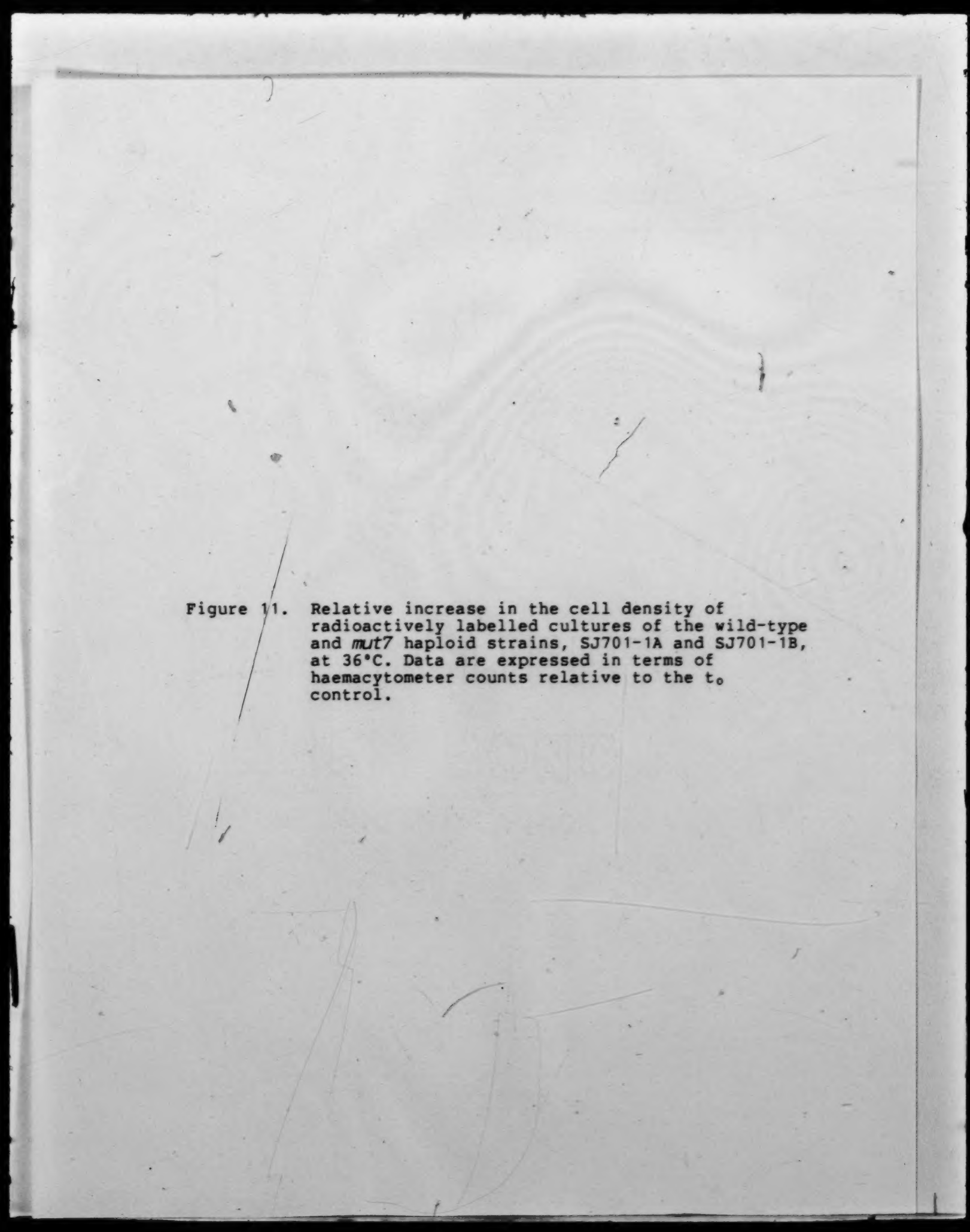
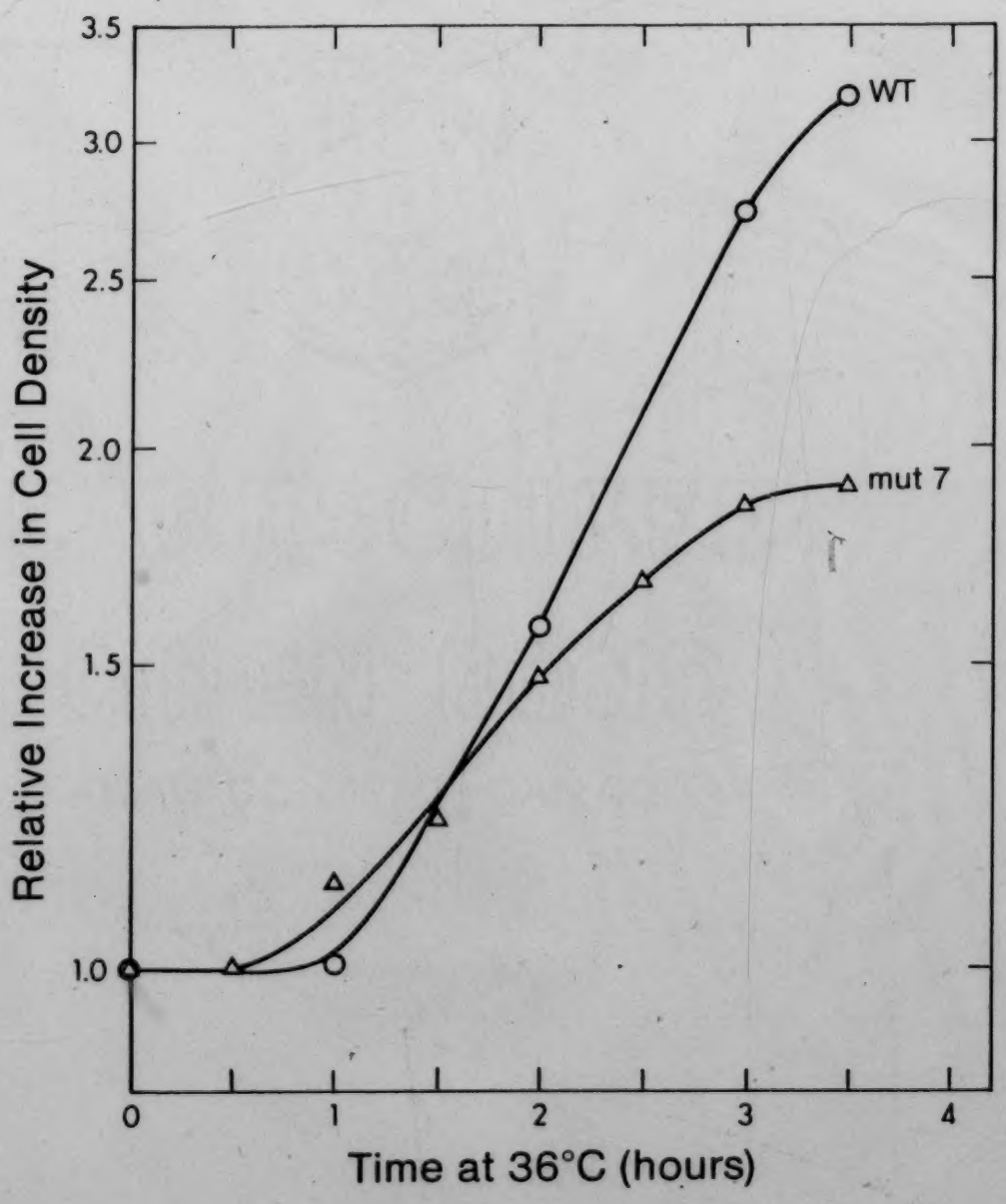


Figure 11. Relative increase in the cell density of radioactively labelled cultures of the wild-type and *mut7* haploid strains, SJ701-1A and SJ701-1B, at 36°C. Data are expressed in terms of haemacytometer counts relative to the t_o control.


3.5
3.0
2.5
2.0
1.5
1.0
0
1
2
3
4
WT
mut 7
Relative Increase in Cell Density
Time at 36°C (hours)

initially increased exponentially, but at a slower rate than wild-type; however, the rate of incorporation into DNA slowed after about 1 1/2 hours and ceased to increased after about 2 hours incubation time in the *mut7* strain at 36°C. A defect blocking the elongation of DNA synthesis would be expected to produce an immediate cessation in the incorporation of counts into DNA. Therefore, the observation that radioactive counts continued to be incorporated into DNA in the *mut7* strain following a shift to the restrictive temperature is not consistent with a defect in the elongation of DNA synthesis. Because total cessation of DNA synthesis was observed to occur following an initial delay, these data were indicative of a defect in the initiation of new rounds of DNA synthesis while allowing rounds of DNA synthesis in progress to procede to completion.

Genetic Analysis of the *mut7* Mutation

The *mut7* mutation complemented the other conditional lethal cell division cycle mutations, *cdc1* through *cdc33*, *cdc40*, *dbf5* and *dbf6*. To ensure that intragenic complementation was not occurring, *mut7* was tested for allelism with mutants of similar phenotype by making generic crosses and observing the segregation of alleles among the dissected spores. Allelism with *cdc2* was tested because of its similarity to *mut7* in both terminal phenotype and execution point; allelism with *cdc4* and *cdc7* was tested because of their similarity to *mut7* in the kinetics of DNA

synthesis at the restrictive temperature (Hartwell, 1973); allelism with *dbf5*, *dbf6*, and *rna6* was tested because, like *mut7*, they are temperature sensitive mutators, however, unlike *mut7*, they are defective in both RNA and DNA synthesis (Johnston and Thomas, 1982a). Since non-temperature sensitive spores were segregated in each of these crosses, *mut7* was found to be non-allelic with these mutations.

In an attempt to map the *mut7* mutation, a *mut7* strain (SJ9-3B) was crossed to a strain carrying the tightly centromere-linked marker, *met14* (SJ24-1A). The diploid strain (SJ25) was sporulated and the segregation of the alleles among the dissected spores was observed. Classification of the tetrads produced by this cross gave the following data: 28 tetratype, 6 parental ditype, and 7 non-parental ditype tetrads (SDS=0.75). Since a mutation is considered to be centromere-linked if it shows an SDS frequency which is significantly less than 0.67, *mut7* does not show centromere linkage.

Spontaneous Mutation Rate Determination

Using the 1000-compartment fluctuation test (von Borstel, 1978), the spontaneous reversion rates for the alleles *lys1-1* (locus revertants and ochre nonsense suppressor mutants) and *his1-7*, in wild-type and *mut7* strains, were determined. Reversion rates at the permissive temperature, 25°C, were compared to those at the

semi-restrictive temperature, 30°C. Survival of *mut7* strains, measured as colony forming ability, decreased only 30% at 30°C, compared to wild-type.

Effect of the *mut7* Allele on Reversion Rates to Histidine Independence. Compartment test data for *mut7* at 25°C showed a slight increase (less than twofold; t_2=3.13, 0.05>p>0.025) over wild-type in production of locus revertants at *his1-7* (Table 6).

At the semi-restrictive temperature, 30°C, compartment data showed a fivefold increase (t_2=7.61, 0.01>p>0.005) over wild-type in production of *his1-7* revertants in *mut7* strains (Table 7).

Comparison of the *his1-7* reversion rates at the permissive temperature, 25°C, to those at the semi-restrictive temperature, 30°C, indicated that the reversion rates do not differ significantly between the two temperatures for wild-type strains (t_2=0.16, p>0.10). However, data for *mut7* at 30°C showed a threefold increase (t_2=7.07, 0.01>p>0.005) over *mut7* at 25°C in production of *his1-7* revertants. Thus, the mutator phenotype of *mut7* was temperature dependent; the mutator activity of *mut7* was enhanced by raising the incubation temperature to the semi-restrictive level.

Effect of the *mut7* Allele on Reversion Rates to Lysine Independence. Compartment test data for *mut7* at 25°C showed an insignificant difference (less than twofold; t_2=1.36, p>0.10) over wild-type in production of ochre suppressor

TABLE 6. Spontaneous reversion rates to histidine independence at 25°C in haploid strains bearing *mut7* or its wild-type allele

Genotype	Strain	Cells/ compt. ($\times 10^6$)	Total no. compts.	No. of revertant compartments: Locus	Mutation rate ($\times 10^{-7}$): Locus
Wild-type	SJ701-1A	1.25	999	287	1.3
	SJ701-1C	1.40	1000	222	0.9
	Average	--	--	--	1.1
mut7	SJ701-1B	1.69	987	417	1.7
	SJ701-1D	1.18	999	363	1.9
	Average	--	--	--	1.8

TABLE 7. Spontaneous reversion rates to histidine independence at 30°C in haploid strains bearing *mut7* or its wild-type allele

Genotype	Strain	Cells/ compt. ($\times 10^6$)	Total no. compts.	No. of revertant compartments	Mutation rate ($\times 10^{-7}$)
				Locus	Locus
Wild-type	SJ701-1A	1.32	1098	347	1.4
	SJ701-1C	1.29	1000	214	0.9
	Average	--	--	--	1.2
mut7	SJ701-1B	1.14	989	691	5.2
	SJ701-1D	0.98	1000	714	6.3
	Average	--	--	--	5.8

revertants for *lys1-1*. On the other hand, data showed a stronger increase (fourfold; t_2=3.89, 0.05>p>0.025) over wild-type in production of revertants at the *lys1* locus itself (Table 8).

At the semi-restrictive temperature, 30°C, compartment data showed a threefold increase (t_2=14.43, 0.005>p>0.0005) in production of *lys1-1* suppressor revertants; however, the strongest effect was observed for the *lys1* locus where revertants increased twelvefold in rate (t_2=6.89, 0.025>p>0.01) (Table 9).

Comparison of the *lys1-1* reversion rates at the permissive temperature, 25°C, to those at the semi-restrictive temperature, 30°C, indicated that reversion rates did not differ significantly between the two temperatures for wild-type strains (locus: t_2=1.0, p>0.10; suppressor: t_2-2.79, 0.1>p>0.05). However, data for *mut7* at 30°C showed a twofold increase (t_2=4.54, 0.025>p>0.01) over *mut7* at 25°C in production of *lys1-1* suppressor revertants and showed a fivefold increase (t_2=5.97, 0.01>p>0.005) over *mut7* at 25°C in production of *lys1-1* locus revertants.

In summary, compartment test data for *mut7* at the semi-restrictive temperature, 30°C, showed an increase over wild-type in production of all types of revertants observed with the strongest effect observed at the *lys1* locus. Furthermore, data for *mut7* at the semi-restrictive temperature, 30°C, showed an increase over *mut7* at the permissive temperature, 25°C, in production of all types of

TABLE 8. Spontaneous reversion rates to lysine independence at 25°C in haploid strains bearing *mut7* or its wild-type allele

Genotype	Strain	Cells/ compt. (x 10^6)	Total no. compts.	No. of revertant compartments		Mutation rate (x 10^{-6})	
				Suppressor	Locus	Suppressor	Locus
Wild-type	SJ701-1A	1.62	1000	69	19	2.2	0.6
	SJ701-1C	1.44	999	44	17	1.6	0.6
	Average	--	--	--	--	1.9	0.6
mut7	SJ701-1B	1.31	1000	108	72	4.2	2.8
	SJ701-1D	1.43	999	67	55	2.3	1.9
	Average	--	--	--	--	3.3	2.4

TABLE 9. Spontaneous reversion rates to lysine independence at 30°C in haploid strains strains bearing *mut7* or its wild-type allele

Genotype	Strain	Cells/ compt. (x 10^6)	Total no. compts.	No. of revertant compartments		Mutation rate (x 10^{-8})	
				Suppressor	Locus	Suppressor	Locus
Wild-type	SJ701-1A	1.76	1000	92	49	2.7	1.4
	SJ701-1C	1.96	989	104	22	2.8	0.6
	Average	--	--	--	--	2.8	1.0
mut7	SJ701-1B	1.23	1000	185	224	8.2	10.2
	SJ701-1D	1.44	1000	196	317	7.5	13.2
	Average	--	--	--	--	7.9	11.7

revertants observed with the strongest effect observed at the *lys1* locus.

DISCUSSION

In the studies presented here, *mut7* was characterized as a conditional lethal cell-division-cycle mutant in order to provide information which would be useful in the identification of the mechanism by which *mut7* increases the spontaneous mutation rate.

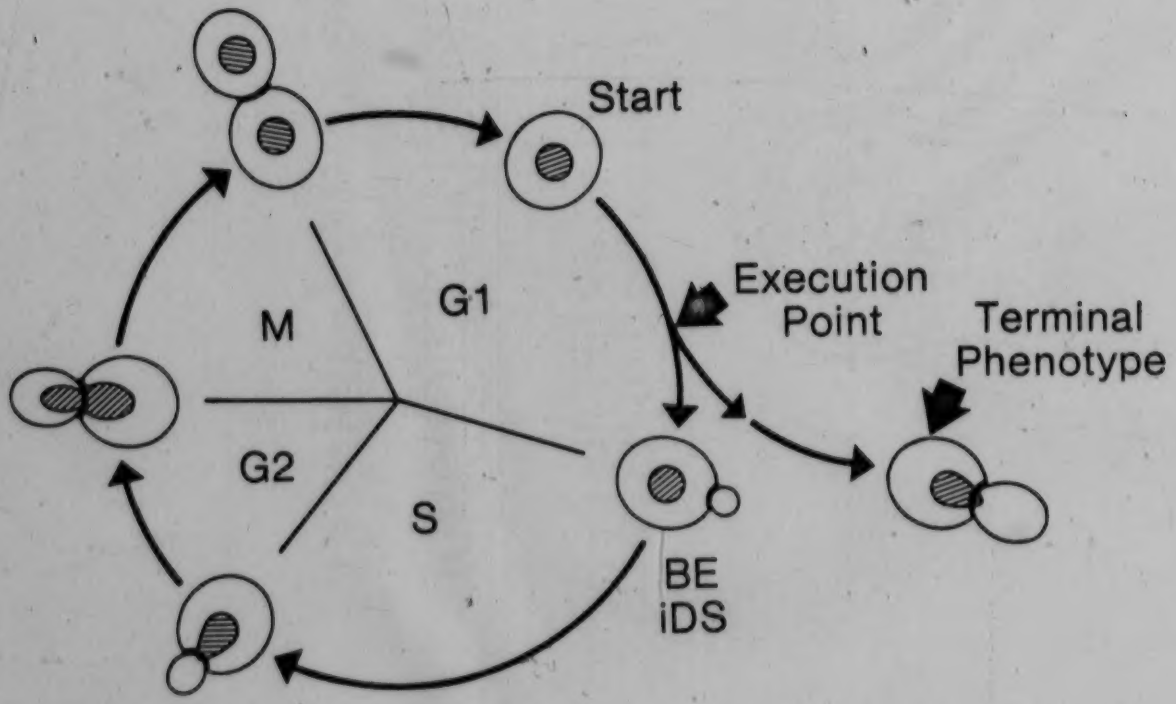
The temperature sensitive phenotype of *mut7* was shown by the decreased survival of *mut7* strains after incubation at the restrictive temperature, 36°C (Figure 2). Growth arrest followed by exponential loss of colony-forming ability in *mut7* strains began after 2 hours at the restrictive temperature, whereas wild-type strains under the same conditions did not show growth arrest (Figure 3). The temperature sensitivity of *mut7* was accompanied by a distinct change in cellular morphology. The proportion of "dumbbell" cells in a *mut7* culture steadily increased with time, whereas the proportion of "dumbbell" cells in a wild-type culture did not increase (Figure 4) following a shift to 36°C. After 6 hours at the restrictive temperature, more than 95% of the cells in a *mut7* culture had collected at the "dumbbell" cellular morphology. Thus *mut7* was shown to be a cell-division-cycle mutant; at the restrictive temperature *mut7* produced morphologically homogeneous cultures which exhibited the "dumbbell" terminal phenotype.

DNA specific fluorescent staining revealed the nuclear morphology of the *mut7* terminal phenotype. A single, unmigrated nucleus was contained in the mother cell of

almost all (83.5%) *mut7* "dumbbell" cells (Figure 5). Other cell-division-cycle mutants which share this terminal phenotype are *cdc2* (Culotti and Hartwell, 1971), which is defective in the initiation of DNA synthesis, and *dbf5* (data not shown). Time-lapse photomicrography enabled the execution point for the completed action of the *mut7* gene product to be determined. The execution point was determined to occur early in the cell cycle, in G1 phase, at about midway between the landmark events of "start" and bud emergence (Figure 6). This position corresponded to the execution point range exhibited by previously identified "dumbbell" *cdc* mutants which are defective in the initiation of DNA synthesis (Hartwell *et al.*, 1973). The cell-division-cycle parameters, terminal phenotype and execution point, of *mut7* are shown with respect to the major landmark events of the *S. cerevisiae* cell cycle in Figure 11.

By measuring the incorporation of a radioactive precursor into nucleic acid *in vivo*, the RNA and DNA synthesis kinetics in cultures of *mut7* and wild-type strains were determined. At the permissive temperature, 26°C, the RNA and DNA synthesis kinetics of the *mut7* and wild-type strains were similar (Figures 7 and 8). At the restrictive temperature, 36°C, the RNA synthesis kinetics of the *mut7* strain were similar to the wild-type strain, whereas the DNA synthesis kinetics of the *mut7* strain were inhibited relative to the wild-type strain (Figures 9 and 10). Thus,

Figure 12. The terminal phenotype and execution point of *mut7* shown with respect to the major landmark events of the *S. cerevisiae* cell cycle. Abbreviations: BE, bud emergence; iDS, initiation of chromosomal DNA synthesis.


Start
Execution
Point
Terminal
Phenotype
M
G1
G2
S
BE
iDS

the *mut7* strain appeared to be specifically defective in a DNA synthesis function.

Determining the cell density of these radioactively labelled cultures allowed the further interpretation of the differences in nucleic acid synthesis kinetics between the *mut7* and wild-type strains at the restrictive temperature. The increase in cell density and the distribution of cellular morphologies in the radioactively labelled wild-type and *mut7* cultures at 36°C (Figure 11; data not shown) were in agreement with observations of unlabelled wild-type and *mut7* cultures at 36°C (Figures 2 and 3; data not shown). Furthermore, the transient inhibition of the increase in cell density which was seen in both wild-type and *mut7* cultures upon shifting to 36°C is consistent with previously published observations (Hartwell, 1973).

The level of incorporation of counts into RNA generally continued to increase exponentially while cessation of cell division was occurring in the *mut7* strain at the restrictive temperature. This observation indicated that RNA synthesis was uninhibited in the *mut7* strain at the restrictive temperature. The level of incorporation of counts into RNA in the *mut7* strain at 36°C increased at a slower rate than in the wild-type strain; however, the slower increase in the cell density of the *mut7* culture compared with the wild-type culture accounts for the observed differences in RNA synthesis kinetics between the wild-type and *mut7* strains at 36°C.

The level of incorporation of counts into DNA initially increased exponentially followed by total cessation of further incorporation after 2 hours in the *mut7* strain at the restrictive temperature. Although the DNA synthesis kinetics of the *mut7* strain at the restrictive temperature, 36°C, were inhibited relative to wild-type, the initial increase in the incorporation of counts into DNA in the *mut7* strain was not consistent with a defect blocking the elongation of DNA synthesis. An elongation defect would be expected to result in the immediate cessation of incorporation of counts into DNA following a shift to the restrictive temperature. On the other hand, an initiation defect, allowing only rounds of DNA synthesis already in progress to procede to completion, would be expected to produce an initial small increase in the level of counts incorporated into DNA. Thus, the observation of an initial increase in the level of incorporation of counts into DNA in the *mut7* strain at the restrictive temperature indicated a defect in the initiation of DNA synthesis.

Using the diphenylamine assay, Ord (1980) observed the abrupt cessation of the increase in total DNA in late log phase cultures of *mut7* strains following a shift to the restrictive temperature. This result would indicate a defect in the elongation of DNA synthesis. An explanation for the discrepancy between Ord's results and the results presented here is not apparent; however, experimental differences such as the growth phase of the cultures and the type of assay

used may be important.

An interpretation of the DNA synthesis data as being indicative of an initiation rather than elongation defect is supported by the cell-division-cycle parameters shown by *mut7* strains. The position of the *mut7* execution point at an early stage of the cell cycle is consistent with the interpretation that this mutant is defective in the initiation of DNA synthesis. The *mut7* execution point was within the range defined by previously studied *cdc* mutants which are defective in the initiation of DNA synthesis (Hartwell *et al.*, 1973). In addition, the terminal phenotype of *mut7* is similar to the terminal phenotype of another *cdc* mutant which is also defective in the initiation of DNA synthesis, *cdc2*. Thus, the terminal phenotype and the execution point of *mut7* are consistent with a defect in the initiation of DNA synthesis.

Determination of the amount of residual DNA synthesis occurring in a *mut7* culture after a shift from the permissive to the restrictive temperature would allow further characterization of the *mut7* initiation defect. A complete or general initiation defect, as in the case of *cdc4* and *cdc7* (Culotti and Hartwell, 1971), would be expected to result in a small amount of residual synthesis associated with the completion of rounds of DNA synthesis already in progress. An incomplete or replicon-specific initiation defect would be expected to result in a larger amount of residual synthesis associated with the continued

replication of a portion of the genome. As an example of this type of mutant, *cdc2* fails to replicate a random fraction (about 1/3) of its chromosomes at the restrictive temperature but once initiation has occurred on a particular chromosome, replication proceeds normally to completion (Conrad and Newlon, 1983). Thus, the amount of residual DNA synthesis occurring in an initiation defective mutant strain under restrictive conditions is indicative of the type of initiation defect present.

From the data presented here, estimation of the amount of residual DNA synthesis occurring in a *mut7* culture after a shift from the permissive to the restrictive temperature may be inaccurate for two reasons. First, the specific activity of the nucleotide precursor pools may vary between wild-type and *mut7* strains at the restrictive temperature, 36°C, making a direct comparison of radioactive counts incorporated into DNA between wild-type and *mut7* cultures invalid. The specific activity problem posed by radioactively labelled cultures could be avoided by using a fluorescence assay for DNA to accurately calculate the amount of DNA in each *mut7* "dumbbell" cell at the restrictive temperature.

Second, because nucleic acid synthesis kinetics were measured in *mut7* strains containing mitochondrial DNA, the level of nuclear DNA synthesis may be overestimated if mitochondrial DNA synthesis is uninhibited in *mut7* strains at the restrictive temperature. In the case of *cdc2*, which

is defective in the initiation of DNA synthesis, studies in strains with mitochondrial DNA led to a 30% overstimate of the level of nuclear DNA synthesis because mitochondrial DNA synthesis was uninhibited at the restrictive temperature (Conrad and Newlon, 1983). The DNA synthesis kinetics from *cdc2* strains are almost indistinguishable form wild-type when mitochondrial DNA is present (Hartwell, 1973), whereas they are inhibited relative to wild-type when mitochondrial DNA is absent (Conrad and Newlon, 1983). In *cdc2* strains lacking mitochondrial DNA the kinetics of incorporation of counts into DNA are similar to the kinetics observed in *mut7* strains containing mitochondrial DNA. At the restrictive temperature, 36°C, both show cessation of further incorporation after an initial exponential increase of about 2 hours duration. This similarity in DNA synthesis kinetics may indicate that mitochondrial DNA synthesis is inhibited in *mut7* strains at the restrictive temperature. However, in order to determine the effect, if any, of *mut7* on mitochondrial DNA synthesis, studies on strains without mitochondrial DNA would have to be compared to the results presented here.

Discussion of a possible role for the *mut7* gene product is limited by the lack of information concerning the mechanism of the initiation of replication in *S. cerevisiae*. Furthermore, procaryotic studies may not provide useful models for the mechanism of initiation of replication in eukaryotes; the organization of eukaryotic DNA into

chromatin and chromosomes shows greater structural complexity than the organization of procaryotic DNA (reviewed in Fangman and Zakian, 1981). Thus, it is difficult to speculate as to what the *mut7* gene product defect may be from these initial studies.

Complementation testing confirmed that *mut7* is not allelic with *cdc1* through *cdc33*, and *cdc40* (von Borstel, unpublished data). Recently isolated *cdc* mutants have not been tested for allelism. These mutants include those which are defective in the "start" event (Reed, 1980), and the collection of cold-sensitive *cdc* mutants, which are defective in the elongation of DNA synthesis (Moir *et al.*, 1982). Because these mutants do not share any phenotypic similarities with *mut7* they are not expected to be allelic with *mut7*. To ensure that intragenic complementation was not occurring, the segregation of alleles in genetic crosses, between *mut7* and mutants with phenotypic similarities to *mut7*, was observed. All alleles tested, including *cdc2*, *cdc4*, *cdc7*, *dbf5*, *dbf6* and *rna6*, were found to segregate independently from the *mut7* allele. Thus, the *mut7* allele appears to define a previously undescribed *cdc* locus.

The spontaneous reversion rates in wild-type and *mut7* strains at two different alleles were measured at the permissive and semi-restrictive temperatures to determine if the mutator activity of *mut7* could be enhanced as the temperature was raised. The reversion rates of wild-type strains derived from the same tetrad as the *mut7* strains

were measured and served as controls; the wild-type spontaneous reversion rates for both alleles tested agree with compartment test data obtained previously (Quah *et al.*, 1980). It was found that the mutator activity of *mut7* was enhanced by raising the temperature to the semi-restrictive temperature, 30°C, from the permissive temperature, 25°C. Under the same conditions, the spontaneous mutation rate of wild-type did not increase significantly. Compartment test data for *mut7* at the semi-restrictive temperature, 30°C, showed an increase over wild-type at the same temperature in the production of all types of revertants observed with the strongest effect observed at the *lys1* locus. Furthermore, data for *mut7* at the semi-restrictive temperature showed an increase over *mut7* at the permissive temperature in the production of all types of revertants observed with the strongest effect being observed at the *lys1* locus. Thus, the spontaneous mutation rate was shown to be enhanced when the *mut7* gene product was partially inactivated.

The approximately wild-type growth rate of *mut7* strains at the permissive temperature, 25°C, may indicate that the mutator phenotype displayed by *mut7* strains at these temperatures results from defective attempts at the initiation of DNA synthesis rather than the failure to initiate. However, it is the failure to initiate DNA synthesis which apparently results in the temperature sensitive cell-division-cycle phenotype of *mut7* strains at the restrictive temperature, 36°C.

Although the effects of the *mut7* allele on the production of *his1-7* and *lys1-1* (locus and suppressor) revertants are small (2- to 4-fold) at the permissive temperature, they agree with compartment test data obtained previously (Table 1; S.-K. Quah, unpublished data). These effects are sufficient to enable the identification of the *mut7* allele when it is segregating in genetic crosses.

The apparent mutational specificity of *mut7* for the production of revertants at the *lys1* locus itself, rather than for the production of ochre nonsense suppressor mutants is difficult to interpret because of the lack of information about locus revertants of the *lys1-1* allele. Because the *lys1-1* allele is suppressed by forward mutations of ochre nonsense suppressor loci which encode genes for tyrosine tRNAs (reviewed in Sherman, 1981), it is possible that locus revertants occur through the mutation of the ochre nonsense codon to a tyrosine codon (i.e. TAA → TAPy; Py = pyrimidine). Thus, it is possible that the apparent mutational specificity of *mut7* for the production of *lys1* locus revertants reflects a preference for the production of AT → CG or AT → TA transversion events (von Borstel *et al.*, 1973). However, it is not known if amino acids other than tyrosine are acceptable at this position in the protein and furthermore, the effects of neighbouring base pairs on reversion rates in *S. cerevisiae* are not known. Thus, without further information about *lys1* locus revertants it is not possible to determine the nature of the mutational

specificity, if any, of the *mut7* allele.

Discussion of the mutator phenotype of *mut7* in light of the cell-division-cycle phenotype of *mut7* may provide information on the mechanism of the *mut7* induced mutator activity. *Mut7* was interpreted to be a *cdc* mutant which is incompletely defective in the initiation of DNA synthesis at the restrictive temperature. It is difficult to imagine how an initiation defective mutant could produce enough spontaneous mutations directly through defective replication to account for such a general mutator effect. Furthermore, it is difficult to imagine how an initiation defective mutant could produce enough lesions to increase the spontaneous mutation rate through the use of a mutagenic repair process. Perhaps the inhibition of the initiation of DNA replication produces a stage where the DNA is particularly susceptible to damage. In this way, *mut7* could indirectly cause the production of many lesions which, if requiring a mutagenic process for repair, could perhaps be numerous enough to account for the increase in the spontaneous mutation rate. Preliminary results which indicate the presence of DNA damage in *mut7* strains include a hyper-recombinogenic phenotype at the permissive temperature, 25°C (Ord, M.Sc. Thesis, University of Alberta, 1980) and difficulty in the isolation of high-molecular-weight DNA at the semi-restrictive temperature, 30°C (S.K. Quah, unpublished observations).

A further possibility is that the *MUT7* gene function may be required for the initiation of accurate DNA repair processes as well as the initiation of chromosomal DNA synthesis. This type of repair deficient mutant would be expected to be sensitive to various DNA damaging agents; however, *mut7* strains are not sensitive to UV or gamma-radiation, and are only moderately sensitive to MMS (Nasim and Brychcy, 1979). Thus, the *MUT7* gene function may be required for the initiation of accurate repair processes following the production of DNA damage by select agents.

A direct approach to the problem of the mechanism(s) of spontaneous mutagenesis, and its enzymology, in *S. cerevisiae* has not been attempted and therefore, this research is currently at the level of general genetic characterization. Because *S. cerevisiae* may share some similarities with procaryotes, the mutator effects of *mut7* will be discussed in relation to mechanisms of spontaneous mutagenesis in *E. coli*.

In response to conditions which damage DNA or inhibit DNA replication, *E. coli* exhibits phenomena, including the enhanced capacity for DNA repair and mutagenesis, which are collectively termed induced SOS error-prone repair (reviewed in Little and Mount, 1982; Witkin, 1976). According to the SOS repair model, the suppression of the fidelity of DNA replication occurs under DNA damaging conditions as an adaptive mechanism to enable translesion synthesis to take place. One of these SOS repair inducing conditions which

inhibits DNA replication is high temperature treatment of a temperature sensitive *dnaB* mutant defective in both the initiation and elongation of DNA replication (reviewed in Witkin, 1976; Caillet-Fauquet and Defais, 1977). The mechanism whereby *dnaB* induces SOS error-prone repair may involve the extensive damage of DNA because degradation of DNA is known to occur in some *dnaB* strains at the high temperature (Buttin and Wright, 1968) and DNA damaging agents are known to induce SOS error-prone repair.

Both *dnaB* of *E. coli* and *mut7* of *S. cerevisiae* are mutator mutants which are also defective in the initiation of DNA synthesis. Thus, *dnaB* is the closest procaryotic analogy to *mut7* at this time. However, the mutator phenotype of *dnaB* may be the result of its elongation defective phenotype rather than its initiation defective phenotype. If *dnaB* and *mut7* share similarities, the mutator activity of *mut7* may be due to an error-prone repair process which is functioning in response to DNA damage caused by the inhibition of the initiation of DNA replication. Because the mutator activity of *dnaB* is dependent on the induction of an error-prone repair mechanism, it is necessary to explore the evidence for the existence of an analogous type of repair in *S. cerevisiae*.

An examination of the SOS error-prone repair model of *E. coli* has been attempted in *S. cerevisiae* through the analysis of UV-induced mutation in mating experiments between irradiated and unirradiated haploid strains

(Lawrence and Christensen, 1982). At least 40% of the UV-induced mutations were found to occur in what was originally the unirradiated nucleus, thus implying a general reduction in the fidelity of DNA replication. Lawrence and Christensen (1982) were unable to find an inducible factor which could be responsible for the reduction in DNA replication fidelity and they therefore, proposed a model of limited fidelity to explain their observations. By this model, untargeted mutations arise in the unirradiated nucleus as a consequence of the saturation of the repair processes which normally maintain fidelity, rather than by the induced reduction in DNA replication fidelity which occurs as an adaptive response to DNA damage in *E. coli*.

Although it is debatable at the present time as to whether the mutagenic repair processes in *S. cerevisiae* are constitutive or inducible, the model of the mutator activity of *mut7*, which has been developed from the interpretation of its cell-division-cycle phenotype, may be accomodated in either case. The main features of this model are that DNA damage, which is caused by the replication block, either requires a mutagenic process for repair (constitutive model) or induces error-prone repair (inducible model), resulting in the enhanced production of mutations distributed throughout the genome.

In order to test this model it will be necessary to identify whether or not DNA damage and untargeted mutagenesis are enhanced in *mut7* strains. Experiments to

determine whether or not DNA damage is enhanced in *mut7* strains would include analysis of the structure of the DNA by alkaline and neutral sucrose gradients to detect strand breakage, and by electron microscopic observation to detect structural abnormalities such as localized denaturation. Furthermore, if DNA damage is enhanced in *mut7* strains, it would be important to correlate an increase in the extent of this damage with the enhanced mutator activity of *mut7* at the semi-restrictive temperature, 30°C. Experiments to determine whether or not untargeted mutagenesis is enhanced in *mut7* strains could be tested through mating experiments which would be analogous to those performed by Lawrence and Christensen (1982). Matings between haploid *mut7* strains where one parent has been pulse treated with the restrictive temperature and then rescued by mating to an untreated parent, would enable the determination of the proportion of untargeted mutagenesis by measuring the reversion rate of a particular allele in the untreated nucleus. Of course, this experimental approach depends on the mutation rate being enhanced by the pulse treatment of one of the parent strains.

In conclusion, *mut7* was characterized in the manner of a cell-division-cycle mutant in order to provide information which would be useful in the identification of the mechanism by which *mut7* increases the spontaneous mutation rate. *Mut7* was found to be defective in the initiation of DNA replication at the restrictive temperature, 36°C. These

results are most compatible with a model of *mut7* acting indirectly in producing spontaneous mutations rather than acting directly through reduced fidelity during DNA replication. Although the question of the mechanism by which *mut7* exhibits its mutator phenotype remains unanswered, the possibility exists that this may be related to its concomitant cell-division-cycle phenotype.

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